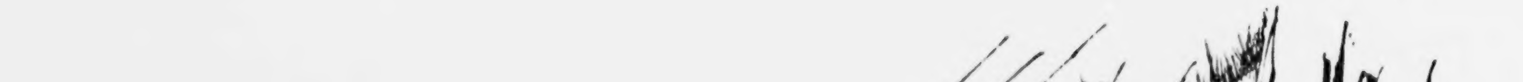
PB191215

CLEAN WATER

WATER POLLUTION CONTROL RESEARCH SERIES ● 14010EGJ11/69
DAST-21

W70-05959

Effect of Antibacterial Agents on Mine Drainages



Reproduced by the
CLEARINGHOUSE
for Federal Scientific & Technical
Information Springfield Va. 22151

U.S. DEPARTMENT OF THE INTERIOR ● FEDERAL WATER POLLUTION CONTROL ADMINISTRATION

149

WATER POLLUTION CONTROL RESEARCH SERIES

The Water Pollution Control Research Reports describe the results and progress in the control and abatement of pollution of our Nation's Waters. They provide a central source of information on the research, development and demonstration activities of the Federal Water Pollution Control Administration, Department of the Interior, through inhouse research and grants and contracts with Federal, State, and local agencies, research institutions, and industrial organizations.

Triplicate tear-out abstract cards are placed inside the back cover to facilitate information retrieval. Space is provided on the card for the user's accession number and for additional keywords. The abstracts utilize the WRSIC system.

Water Pollution Control Research Reports will be distributed to requesters as supplies permit. Requests should be sent to the Publications Office. Dept. of the Interior, Federal Water Pollution Control Administration, Washington, D.C. 20242

Effect of Antibacterial Agents on Mine Drainages

Use of Viable Antibacterial Agents to Reduce Pollution by Mine Drainages

by
Robert E. Shearer
William A. Everson

FEDERAL WATER POLLUTION CONTROL ADMINISTRATION

DEPARTMENT OF THE INTERIOR

by

MSA Research Corporation
Evans City, Pennsylvania

for

COMMONWEALTH OF PENNSYLVANIA
DEPARTMENT OF MINES AND MINERAL INDUSTRIES

Grant 14010EGJ

November 1969

FWPCA Review Notice

This report has been reviewed by the Federal Water Pollution Control Administration and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Federal Water Pollution Control Administration.

TABLE OF CONTENTS

Section No.		Page No.
1	Abstract	iii
2	List of Figures	v
3	List of Tables	ix
4	Conclusions	1
5	Recommendations	3
6	Introduction	5
7	Construction Design of Experimental Apparatus and Procedures	9
8	Isolation and Identification of Iron and Sulfur Bacteria from Test Sites	11
9	Behavior of Natural Inhibitors	17
10	Field Tests in Robena Mine with Natural Inhibitors	39
11	Attempts to Isolate, Concentrate and Identify Phages	45
12	Caulobacters	57
13	Streptomyces as Producers of Antibiotic Inhibitors	91
14	Acknowledgements	115
15	References	117
16	Glossary	119

Appendix No.		
1	Statistical Analysis of Inhibition in Various Streams	121
2	Analytical Data on Horizontal Flooded and Vertical Spray Systems	123

SECTION 1

ABSTRACT

A program was carried out to characterize the nature of the active agents in certain natural waters that had previously been found to inhibit bacterial production of acid in streams through submerged piles of coal in plastic containers in the laboratory. A further minor program was undertaken to test the feasibility of application of the inhibitory principle to an actual coal mine situation where acid was being produced.

In the mine study, two (2) inoculations of the natural water previously found to be inhibitory were made in the amount of about 14,000 gallons each to a worked-out region of Robena mine in Greene County, Pennsylvania. After the first inoculation was made, laboratory tests showed absence of inhibitory power in the water at the time it was collected; and the water used for the second test was similarly found to be only weakly inhibitory. No effect was noted in the drainage of the test site over periods of several months after each inoculation. In addition to the possibility of the weakness or absence of the inhibitory principle, it is now believed that poor topography of the floor of the mine of the test site might render application of inhibitor ineffective. A more promising test site is believed to be at Karen mine, also in southwestern Pennsylvania.

In efforts to characterize the nature of the inhibitor, earlier, unreplicated, laboratory experiments were duplicated on streams through submerged acid-producing piles of coal in plastic containers. Inoculation of both raw and treated waters previously found inhibitory resulted in a decrease in production of acid, dissolved iron and sulfates and in an increase in pH.

Strains of Caulobacter which are bacteria characterized by stalked appendages were found in the natural inhibitory waters; and when adapted to acidic environments and concentrated in culture media, they were inoculated into laboratory coal piles. They then induced inhibition of the acid production. Other adapted strains of Caulobacters obtained commercially also induced inhibition. Some evidence was shown that Caulobacter inhibitors might move downstream in a flooded horizontal stream through piles of coal refuse producing acid.

A group of sensitivity disks containing antibiotics made by various species of Streptomyces was screened for effectiveness against the iron-sulfur bacteria producing acid in mines. The four (4) species of Streptomyces producing the antibiotics found to be effective were adapted to the acidic conditions found in mine waters and were found effective against the acid-producing bacteria in test tube cultures and on solid media.

SECTION 2

LIST OF FIGURES

Figure No.		Page No.
1	Flooded Vertically Flowing System	18
1a	Flooded Vertically Flowing System-Detail	19
2	Effect of Inhibitor Upon Production of Sulfate and Acid by Karen Bacteria	23
3	Ferrous and Total Iron by Karen Bacteria with Filtrate Inhibitor	24
4	Ferrous and Total Iron Production by Uninhibited Karen Bacteria	25
5	Ferrous and Total Iron Production by Karen Bacteria with Raw Inhibitor	26
6	Effect of Inhibitor Upon pH in Karen Cultures	27
7	Oxidation Times for Karen Bacteria	27
8	Inhibitors for Ferrous Iron Production by Robena Bacteria	28
9	Effect of Inhibitor on Production of Total Iron in Robena Cultures	29
10	Oxidation Times of Robena Bacteria	30
11	Effect of Inhibitor on pH of Robena Cultures	30
12	Effect of Inhibitor on Sulfate Production by Robena Bacteria	31
13	Effects of Inhibitors on Acid Production by Robena Bacteria	31
14	Flooded Horizontally Flowing System	33
14a	Flooded Horizontally Flowing System-Detail	34
15	Vertical Spray Systems - Not Flooded	36
15a	Vertical Spray Systems - Not Flooded-Detail	37

LIST OF FIGURES (cont'd)

Figure No.		Page No.
16	Locations of Inhibitor Injection Point and Drainage Pickup Points in Robena Mine	40
17	Zones of Inhibition on Agar Layers and Electron Micrograph of Caulobacters	60
18	Stock and McCormick Adapted Caulobacter Cultures - Standard Agar Plates	62
19	Adapted Caulobacter Cultures Used to Inoculate Coal	69
20	Two Electron Micrographs of Predominant Bacteria in Effluents from Caulobacter - Inhibited Systems	71
21	Effect of Caulobacter on Production of Ferrous Iron by Robena Bacteria - Test 2	76
22	Effect of Caulobacter on Production of Ferrous Iron by Robena Bacteria - Test 1	76
23	Effect of Caulobacter on Production of Total Iron by Robena Bacteria - Test 1	77
24	Effect of Caulobacter on Production of Total Iron by Robena Bacteria - Test 2	77
25	Effect of Caulobacter on Sulfate Production by Robena Bacteria - Test 1	78
26	Effect of Caulobacter on Sulfate Production by Robena Bacteria - Test 2	78
27	Effect of Caulobacter on Oxidation Times of Robena Bacteria - Test 1	79
28	Effect of Caulobacter on Oxidation Times of Robena Bacteria - Test 2	80
29	Effect of Caulobacter on Acid Production by Robena Bacteria - Test 2	80

LIST OF FIGURES (cont'd)

Figure No.		Page No.
30	Effect of Caulobacter on Acid Production by Robena Bacteria - Test 1	81
31	Effect of Caulobacter in pH of Cultures of Robena Bacteria - Test 1	82
32	Effect of Caulobacter in pH of Cultures of Robena Bacteria - Test 2	83
33	Effect of S. aureofaciens on Oxidation Times of Robena Bacteria	113
34	Effect of S. aureofaciens on pH of Robena Cultures in Upward-Flowing Vertical Systems	114
35	Effect of S. aureofaciens on Acid Production by Robena Bacteria on Upward-Flowing Vertical Systems	114

SECTION 3

LIST OF TABLES

Table No.		Page No.
1	Liquid Media Formulations (1)	12
2	Solid Medium - Thiosulfate Agar	12
3	Solid Medium - Iron Agar (1)	13
4	Bacterial Population of Test Site Drainages	15
5	Additions of Inhibitor to Cultures of Robena and Karen Bacteria	17
6	Viability of Karen Bacteria	21
7	Viability of Robena Bacteria	22
8	Dissolved Oxygen Content of Various Streams	32
9	Dissolved Oxygen (ppm) at Various Ports in Horizontal Systems	35
10	Addition of Inhibitors to Vertical Spray Systems	38
11	Volumes of Trace Water and Inhibitor Introduced Into Robena Mine	41
12	Analyses of Water From - 11 Right	42
13	Analyses of Water From - 12 Right	43
14	Drainage Sources as Filtrates Against Iron Bacteria	49
15	Testing Adapted Caulobacters Against Iron Bacteria	64
16	Steps in Attempts to Adapt Caulobacters	66
17	Viability of Robena Bacteria in Vertical Streams With and Without Caulobacter Inhibitor	74

LIST OF TABLES (Cont'd)

Table No.		Page No.
18	Effects of Caulobacters Along Horizontal Systems	88
19	Antibiotic Sensitivity Disk Testing	92
20	Testing Antibiotic Producer Products Against Iron Bacteria	96
21	Inhibitory Effect of Coal-Grown Streptomyces (Sucrose Added) (16 days)	99
22	Inhibitory Effect of Coal-Grown Streptomyces (Sucrose Added) (21 days)	101
23	Inhibitory Effect of Coal-Grown Streptomyces (No Additives) (17 Days)	104
24	Inhibitory Effect of Coal-Grown Streptomyces (No Additives) (25 Days)	105
25	Inhibitory Effect of Coal-Grown Streptomyces (No Additives) (35 Days)	106
26	Analyses of Streptomyces - Inhibited Tubes	108
27	Testing for Presence of Streptomyces - Induced Iron Reduction	110
28	Viability of Robena Bacteria in Systems Inoculated with S. aureofaciens	111
29	Viability of Robena Bacteria in Systems Inoculated with S. spheroides	111
30	Test Data on Streptomyces as Inhibitor	112
31	Statistical Data on Characteristics of Inhibited Streams	122
32	Total Acidity (ppm $CaCO_3$ equiv.) Along Horizontal System	124
33	Total Iron Along Horizontal System	125

LIST OF TABLES (Cont'd)

Table No.		Page No.
34	Ferrous Iron Along Horizontal System	126
35	Oxidation Times of Bacteria From Along Horizontal System (days)	127
36	pH Along Horizontal System	128
37	Sulfates Along Horizontal System (ppm)	129
38	Total Iron (ppm) Through Vertical Spray System	130
39	Ferrous Iron (ppm) Through Vertical Spray System	132
40	Sulfates (ppm) Through Vertical Spray System	134
41	pH Through Vertical Spray System	136
42	Total Acidity Through Vertical Spray System (ppm)	138

SECTION 4

CONCLUSIONS

Replicate tests confirmed presence of agents which inhibit production of ferrous sulfate from pyrites by bacteria in an upward-flowing flooded vertical system observed in an earlier project in unreplicated tests on McCormick mine effluent and in waters from the region of Wildwood mine.

No proof was found of inhibition by unconcentrated unoptimized natural inhibitor introduced into the bottom of downward-flowing unflooded vertical systems, thus the inhibitor could not be demonstrated to move vertically in an unsaturated system.

No proof was found of inhibition by normal quantities or copious quantities of unconcentrated unoptimized natural inhibitor introduced into the influent of a flooded downward flowing horizontal system.

A species of Caulobacter was isolated from the McCormick mine effluent and when cultured in higher titer was found to inhibit bacterial action on pyrites to form ferrous sulfate.

Other strains of Caulobacters obtained from the American Type Culture Collection were found to inhibit acid production in flooded upward-flowing vertical systems. In an unreplicated test, possible evidence of downward movement of inhibition by Caulobacters on acid-producing bacteria was seen in a flooded downward-flowing horizontal system.

An adapted strain of antibiotic-producer (S. aureofaciens) was found to inhibit acid production in replicate static tests and in flooded upward-flowing vertical systems.

SECTION 5

RECOMMENDATIONS

Four (4) promising strains of Caulobacters should be studied as inhibitors of acid-producing bacteria in upward-flowing flooded vertical systems, which have been shown to be susceptible to inhibition in the past. The most effective strain from this and from two (2) strains already studied should then be studied as inhibitors in flooded horizontal streams and in unflooded downward-flowing vertical streams when introduced into the bottom, since these systems seem to be the most difficult to obtain inhibition.

One antibiotic-producing strain of Streptomyces (S. aureofaciens) was adapted to acid mine conditions. This Streptomyces inhibited acid iron bacteria in replicate in static tests and in upward-flowing vertical flooded systems. This adapted microorganism should also be studied as an inhibitor under simulated mine conditions as given above. If successful, its effects on aquatic life such as plants, fishes and the like should be studied.

The most effective inhibitor should be inoculated into a wide range of acid-producing regions in mine workings, including settling basins for fines from coal washings, caved-in underground workings through boreholes and into high points in these workings and over refuse piles with acid runoff.

SECTION 6

INTRODUCTION

This is the final report on a project which was initiated in July, 1968 and extended into the last half of 1969.

The objectives of this project were:

1. To extend laboratory results to test the feasibility in coal mines of reducing the production of acid in drainages.
2. To characterize in the laboratory the nature of the inhibitory material.

The facilities of the Robena and Karen mines of United States Steel Corporation were made available for this project. A truckload of coal from Robena mine was furnished for experiments, samples of mine effluent containing acid-producing bacteria were furnished from both mines, and a site where acid was being formed was fitted out with plumbing to introduce inhibitors for field tests. The background for this project and details of tests are discussed in a later section.

MSA Research Corporation had conceived the idea that application of viable anti-bacterial agents to acid-producing regions in coal mines might reduce the pollution in the effluents of these regions. The Coal Research Board of the Commonwealth of Pennsylvania supported two (2) programs in which a widespread search for natural inhibitors was undertaken (Shearer, R.E. et al 1968). Emphasis was first placed on bacteriophages, or viruses which are specific for a given species of bacteria. However, any viable agent antagonistic to acid-producing bacteria which would proliferate in the mine waters would be suitable. The search for inhibitors covered materials such as compost, sewage, muds, neutral effluents from many mines and the like. A new solid medium described in Section 8 had to be developed for assay of the acid-Producing bacteria. Effective inhibitors were found in the effluent of McCormick mine, Butler County, Pennsylvania and in a region of Pine Creek, at the bottom of a refuse pile at Wildwood mine, Allegheny County, Pennsylvania. The effects of the inhibitors in lowering acidity and raising pH were observed in effluents from flooded vertical beds of coal in the laboratory as well as in static liquid and solid culture. It was found that these natural inhibitors varied in effectiveness from time to time and that the nature of the inhibiting agent had to be established to insure dependability. Some evidence was developed that one (1) of the inhibitory agents might be a species of Caulobacter.

More recently, the present program supported jointly by the Pennsylvania Coal Research Board and the Federal Water Pollution Control Administration was undertaken to characterize the nature of the inhibitor and to apply inhibitor to coal mines that are producing acid. The first attempt to apply natural inhibitor to a mine was unsuccessful. This may well be due to the seasonal variability in the source of inhibitor since a sample of this source drawn at the time the dose was collected failed to show inhibition in the laboratory. The nature of inhibitor had not then been established. Another possibility is that the topography of the floor of the mine is such that inhibitor did not flow into the acid-producing regions. Another attempt was made with Wildwood water containing the inhibitor after it had been found to have become effective again. No inhibition was noted in the mine effluent in early checks; but monitoring continues.

Replication of tests of natural inhibitor through flooded vertical coal piles confirmed evidence of inhibition. A study of movement of natural inhibitor showed no transfer of natural inhibitor upward against a light stream flowing downward through unsaturated coal piles nor horizontally through a flooded horizontal bed of coal refuse. Horizontal movement of a stock Caulobacter inhibitor was suggested in a test which remained unreplicated because of depletion of funds.

The suggestion that a species of Caulobacter was inhibitory was partially confirmed in static laboratory tests and in streams through vertical flooded beds of coal. Optical and electron microscopy confirmed the presence of a species of Caulobacter in the natural inhibitory waters of McCormick mine. There remains to be determined the most effective species of Caulobacter and its characteristics.

A battery of 15 antibiotics was tested against all species of mine acid-producing bacteria and four (4) were found effective against all of these. The various species of Streptomyces bacteria which produce these effective antibiotics under specialized conditions were tested first on static laboratory cultures of acid-producing bacteria and were found effective. Testing on flooded vertical streams is still underway. The relative effectiveness of these species and of the Caulobacter species under various conditions needs to be determined to establish the optimum inhibitor.

With establishment of the optimum inhibitor, tests of its effectiveness in actual mine conditions should be undertaken. Sites are available for such tests.

SECTION 7

CONSTRUCTION DESIGN OF EXPERIMENTAL APPARATUS AND PROCEDURES

Three different systems were designed to study inhibition of acid producing bacteria in flowing streams in coal piles. One was an upward-flowing vertical spray system, one an unflooded downward-flowing vertical spray system, and a downward-flowing flooded horizontal system. These systems are described below. Inhibitors were tested either as filtrates, as found naturally or in synthetic cultures. Filtrates were prepared by introducing one volume of either liquid cultures of iron bacteria from Karen and Robena mine effluents into an equal volume of raw water from the Wildwood mine. The bacteria were incubated for 24 hours, inhibitor added, and the solution incubated an additional 24 hours, then filtered through a 0.2 micron millipore filter.

Upward-Flowing Flooded Vertical Systems

Seven containers, each with a capacity of about 20 liters and measuring 18 1/2" x 11 1/4" x 11 1/4", were set up to study inhibition in upward-flowing flooded vertical systems. These containers were filled with sterilized run-of-mine coal or coal refuse and with 9 liters of filtered tap water. Chemical analyses showed the following composition: sulfur 2.2%, ash 10.5% and Fe_2O_3 24.7%. Stainless steel tubes were inserted into the coal so that filtered tap water could be passed into the bottom of the pile. An outlet was arranged so that an effluent would pass out near the top of the pile. A photograph of the setup is shown in the section on experimentation as Fig. 1. Alkali was first leached out of the coal by passing dilute sulfuric acid through the piles. When leaching was completed, as indicated by stabilization of pH at a value of 5, acid-producing bacteria from Robena and Karen mine effluents were inoculated into the systems and allowed to propagate. When acidity developed, the test containers were considered to be ready for inoculation of inhibitors.

Flooded Downward-Flowing Horizontal System

In practical application of inhibitors to mine drainage systems, it might be desirable to introduce inhibitor to the influent stream of an acid-producing region and then have the agent move throughout the region. To determine if inhibition might be obtained under these conditions, a set of troughs was set up having an input of filtered tap water at one end and an overflow at the other end. The troughs measure 10 feet long, 1 1/2 feet wide and 8 inches deep were filled with sterilized coal. Water (43 gallons) was added to a depth of 4 inches, the height of the overflow. A photograph is shown in the experimental section as Fig. 14. Alkali was leached out of the coal with dilute sulfuric acid. The cultures of acid-producing bacteria were added at the influent and allowed to propagate. When acid was developed, the systems were considered to be ready for introduction of inhibitor.

Downward-Flowing Unflooded Vertical Systems

Some acid-producing regions in mines have water dripping from the roof and walls. In these cases it would be desirable to introduce the inhibitor into the water pool on the floor of the mine and have the inhibitor move upward to the source. Seven (7) plastic containers were fitted out to simulate this condition. These containers measure 42 inches high and 15 inches in diameter and have sample ports installed 2 and 5 inches from the bottom and at intervals every 6 inches above the 5 inch point. The bottom port acted as the discharge point for the acidic water. Spray nozzles were installed above the top and adjusted to spray over the top of the coal at a rate of 2 ml/min. A photograph of the setup is shown in the experimental section as Fig. 15. Sterilized coal was loaded into the containers. When the coal had been leached of alkali with dilute sulfuric acid, cultures of acid-producing bacteria were introduced and allowed to propagate. When an acid discharge developed, the systems were considered to be ready for introduction of inhibitor.

Design of Schedule for Chemical Analyses of Various Systems

Standard methods were designed into a routine analytical schedule for monitoring acid-producing systems. These include the ortho-phenanthroline colorimetric method for dissolved ferrous iron. Total dissolved iron was also determined by this method using hydroxylamine to reduce ferric iron to ferrous before adding the reagents. (Standard Methods for Examination of Water and Waste Water, p-156 (1965)).

The Sulfa-Ver turbidimetric method (Hach Chemical Co.) was used for sulfates, and acidity was determined by titrating hot solution with standard base to a pH of 8.3 using a pH meter to determine endpoint.

SECTION 8

ISOLATION AND IDENTIFICATION OF IRON AND SULFUR BACTERIA FROM TEST SITES

Introduction

Since inhibitors were to be used against the autotrophic iron and sulfur bacteria from test sites at Robena and Karen mines, it was first necessary to identify the species of iron bacteria and to determine to what extent these autotrophic acid-producing bacteria were present at the test mine sites. Acid drainages were collected and analyzed within a few hours after collection to minimize any changes in the contents.

Identification Procedure

Aliquots of acid drainages from Robena and Karen mine sites were inoculated into three (3) types of sterile liquid synthetic iron media, and into thiosulfate and sulfur media to identify the specific iron bacteria present. The compositions of three (3) Fe II liquid media are given in Table 1. These formulations are used as a partial test for identifying the autotrophic iron and sulfur bacteria. The original inoculation ratio was 1:10 drainage to medium. This was followed by a secondary transfer of 1:100 after apparent bacterial growth was evident as noted by ferrous iron oxidation, and/or oxidation of thiosulfate and sulfur. The secondary transfer at higher dilution was necessary so as to be certain bacterial, and not chemical,action was involved. After oxidation of media from the secondary transfer, aliquots were plated on solid media, using iron and thiosulfate agars. The compositions of these media are given in Tables 2 and 3. The composition for liquid thiosulfate is the same as given in Table 2 except that agar was omitted. The pH was 4.5.

The formulation for the sulfur medium is given by Churchill and Leathen, p-18 (1961). The final pH was 4.0. These media are used to biochemically identify the species of iron or sulfur microorganisms present. Ten (10) neutral and alkaline drainages were obtained from various mine areas within the Robena mine complex and tested qualitatively for the presence of iron and sulfur-oxidizing bacteria. It was found that iron bacteria, and, in some cases sulfur bacteria were present, even in drainages with a pH as high as 7.8.

Since these neutral or alkaline drainages contained iron bacteria it appeared unlikely that they would act as inhibitors. However, they were tested as potential iron bacteria inhibitors as described on page 47 .

TABLE 1 - LIQUID MEDIA FORMULATIONS (1)

Compound	Synthetic I	Synthetic A	Synthetic B
$(NH_4)_2SO_4$	0.30 g	0.30 g	0.30 g
KCl	0.10 g	0.10 g	0.10 g
$MgSO_4$	6.72 g	1.00 g	1.00 g
K_2HPO_4	0.10 g	0.10 g	0.10 g
$CaCl_2$	1.94 g	0.22 g	0.22 g
l-tryptophane	----	0.02 g	----

(1) All media are made to 2 liters. The pH is then adjusted to 3.9 with dilute H_2SO_4 and 20 ml of 10% $FeSO_4$ solution are added to A and B. Fifty ml is added to I. The formulations are our own modifications designed to maintain good growth of iron bacteria and simulate actual acid mine drainage.

TABLE 2 - SOLID MEDIUM - THIOSULFATE AGAR

Compound	g/Liter	Preparation
KH_2PO_4	3.00	The base medium was adjusted to pH 4.5 using H_2SO_4. The medium was autoclaved. An aqueous solution of 10 per cent $Na_2S_2O_3 \cdot 5H_2O$ was filter sterilized. Fifty ml of the $(Na_2S_2O_3 \cdot 5H_2O)$ was added to each 950 ml of base medium, which was poured into plates.
$(NH_4)SO_4$	0.20	
$CaCl_2$	0.20	
$MgSO_4 \cdot 7H_2O$	0.10	
Ion agar	8.50	
Distilled Water	1000 ml.	

TABLE 3 - SOLID MEDIUM - IRON AGAR (1)

Component A
Agar Salts

Compound	g/liter	Preparation
$(NH_4)_2SO_4$	0.30	Adjust to pH 3.5 with H_2SO_4. Autoclave.
KCl	0.10	
K_2HPO_4	0.10	
$MgSO_4$	2.00	
$CaCl_2$	0.22	
Component B Ferrous Iron		
$FeSO_4 \cdot 7H_2O$	4.91	Dilute to 300 ml, adjust to pH 3.5 with H_2SO_4 and Morton Filter to sterilize.
Component C Agar		
Ion agar No. 2	8.00	Make slurry in 150 ml H_2O, heat, then autoclave.

Mix 500 ml of A, 300 of B and 150 of C, then pour into sterile Petri dishes. Plates last for 3 or 4 days.

(1) This formula is our own modification and supports good growth of iron bacteria.

Bacteria from the acid drainage of Robena area were identified as the iron bacterium Ferrobacillus sulfooxidans only, while both the iron bacterium, F. sulfo, and the sulfur bacterium Thiobacillus thiooxidans were present in drainage from Karen.

Three (3) types of liquid from media were used for isolation and partial identification of iron oxidizing bacteria. Drainages differed in the types of liquid iron medium oxidized and the speed of oxidation. Synthetic I is a complex medium simulating acid drainage to some extent. Some changes were made in the salt concentration in hopes of providing a better ionic environment for bacteriophage attachment to the iron bacteria. A reference to this can be found in Adams, pp 141-144 (1959). Medium A does not contain as many elements and differs from B only in that A contains a trace of l-tryptophane, an organic material. Bacteria isolated from acid drainage from Robena mine area oxidized I and B, then A slowly. Iron bacteria are adapted to the environment within a particular mine. Different mines, and even different areas within any one (1) mine have differences in the environment. The iron bacteria from various mines will oxidize iron more or less rapidly in various synthetic iron media according to their ability to adapt to the nutritional composition of each medium. This is the reason why ferrous iron media of various compositions were used. An analogy can be made between isolating bacteria from mines and soil in which no one (1) medium is adequate for all species. This is pointed out by Alexander, p-21 (1967).

L-tryptophane was added to medium A in order to determine if iron bacteria could tolerate this organic material. Initially the iron bacteria grew slowly in medium A, but subsequent transfers showed that the rate of iron II oxidation was increased as the iron bacteria adjusted to this medium. The l-tryptophane was also used as some bacteriophages require this material in the medium to provide attachment to host bacterial cells. (Adams, p-145 (1959) and Bradley, p-258 (1967)).

Bacterial Population of Test Site Drainages

Total cell counts were made on the original acid drainages from Karen and Robena. The results are shown in Table 4.

TABLE 4 - BACTERIAL POPULATION OF TEST SITE DRAINAGES

Ident. No.	Drainage	Total Bacteria/ml
1	Karen - Discharge	2.5×10^7
2	Karen - Fox Opening	3×10^7
3	Robena - Whitely	2×10^7
4	Robena - Jenny Room	2.5×10^7

These total bacteria counts may be high in comparison with those given by other investigators. A thorough investigation of the literature was not made, but other investigators used different mine areas and waters for determining total cell counts.

There are undoubtedly many reasons for differences in total cell counts. The total cell count is at best a semi-quantative test and is of little significance since both living and dead bacterial cells are counted. Changing conditions of water flow, sampling sites, temperature, exposed coal surfaces, period between sampling and counting, size and air space in collecting containers, all influence the total numbers of bacteria.

These studies were not greatly concerned with determination of presence of bacteria other than iron and sulfur oxidizers at the Robena and Karen test site. These drainages had pH values averaging from 2.5 to 3.5. At these low pH levels very few other types of bacteria can live. References to this can be found throughout the literature, particularly in Alexander, (1967), and Stanier, et al (1961). Thus the purpose of these studies was to find bacterial inhibitors effective against the iron and sulfur bacteria from the test sites and not to study the complete flora of the waters.

One (1) test was conducted in order to determine if Caulobacters were present in acid drainages from Robena and Karen test site areas. Caulobacters were not found.

Bacterial Population of Wildwood Area

Total bacterial cell counts were not made on the Wildwood and McCormick waters. The pH averaged about 7.0 when samples were collected. Samples were collected along stream soil banks indicating that high numbers of many kinds of microorganisms would be present at this pH. Culturing and identifying all these microorganisms would be an impossible task since conventional microbiological techniques can only estimate a portion of the total number of bacteria. A reference to this can be found in Alexander, p-21 (1967).

The cell numbers are undoubtedly many times higher than those in the acid drainages from Robena and Karen mines since a count of only viable cells from the neutral mines showed on the order of 10^7 cells per ml. The viable counts were made only on one type of medium (standard agar for Caulobacters, Section 12). Use of other media more optimal for enumeration of many types of soil and water bacteria would give much higher viable cell counts, and thus still higher total cells counts since both living and dead bacterial cells are counted.

Chemical analyses were made on Wildwood water at one time when the water was found to have a low pH. This sample is not typical as most waters from this area were neutral. The following values were obtained: pH = 4.5, acidity = 37 ppm $CaCO_3$ equivalent, sulfate = 150 ppm, total iron = 1 ppm, undissolved solids = 2800 ppm.

Chemical analyses were made at various times at the Robena test sites and results given in Section 10.

SECTION 9

BEHAVIOR OF NATURAL INHIBITORS

Unconcentrated raw waters from the Wildwood and McCormick mines were tested against various acid-producing strains of bacteria in streams through horizontal flooded systems, in a vertical system with a light stream of tap water flowing downward, and as a replicate of tests carried out in earlier projects in streams through flooded vertical systems. These were the waters that had shown inhibitor action in earlier studies. Filtrates of these inhibitors were also tested in similar streams. The various tests are discussed below.

Karen and Robena Mine Bacteria in Flooded Upward-Flowing Vertical Systems

In order to replicate tests of inhibition of acid-producing bacteria from Karen and Robena mines performed in an earlier project, Karen bacteria were inoculated into three upward-flowing vertical submerged coal pile systems (numbers 1-3), Robena into three others (numbers 4-6), and the seventh remained as a sterile control. One of the systems is shown in Figures 1 and 1a. When the bacterial populations were observed by chemical and microbiological methods to be thriving as indicated by viable cell counts and maintenance of low pH, one of each of the piles seeded with Robena and Karen bacteria was inoculated with the raw inhibiting water and one each with the inhibitor filtrate. Reinoculations were also made according to Table 5.

TABLE 5 - ADDITIONS OF INHIBITOR TO CULTURES OF ROBENA AND KAREN BACTERIA

No.	Type & Amount of Inhibitor	Days After First Addition	Strain of Bacteria
2	100 ml raw	0	Karen
2	100 ml raw	27	Karen
3	100 ml filtrate	0	Karen
5	1000 ml raw	0	Robena
6	850 ml filtrate	0	Robena
6	100 ml filtrate	25	Robena
6	100 ml filtrate	52	Robena

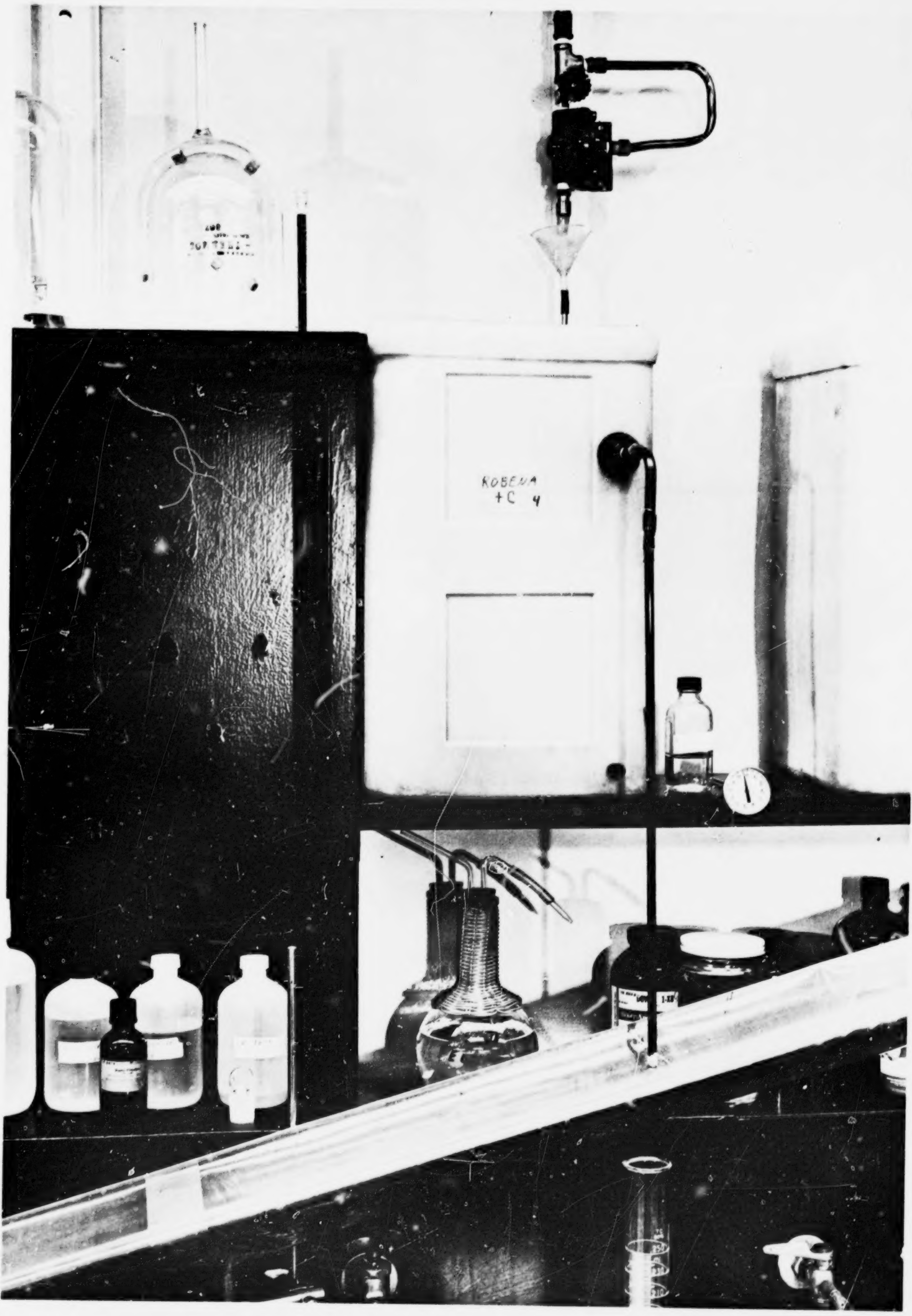


FIGURE 1 FLOODED VERTICALLY FLOWING SYSTEM

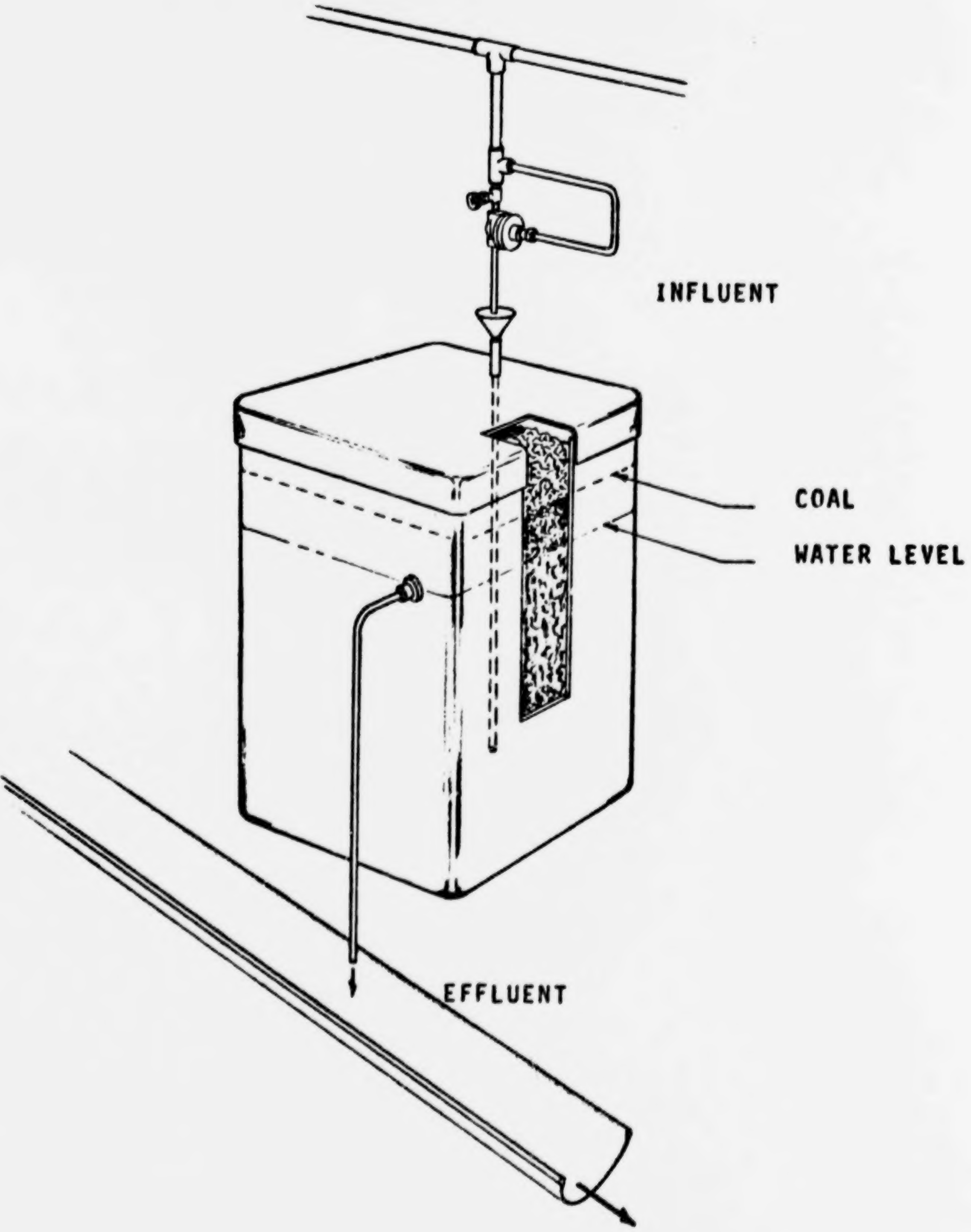


FIGURE 1a FLOODED VERTICALLY FLOWING SYSTEM-DETAIL

One of each pair of inhibitors was raw source as obtained from the selected site at Wildwood mine. One of each pair of inhibitors was similarly a filtrate obtained by introducing one volume of either Karen or Robena liquid bacteria cultures into an equal volume of raw Wildwood drainage. The bacteria were incubated for 24 hours, inhibitor added, and the solution incubated an additional 24 hours, then filtered through a 0.2 micron millipore filter.

The effects of the conditions are shown in Figures 2 through 13 and Tables 6 and 7. Oxidation times refer to the time for liquid iron nutrient media to become cloudy. This indicates bacteria activity compared to controls, which usually takes 2 days to become cloudy. Viabilities are determined by culturing bacteria from dilutions of effluent on solid plates and counting the number of colonies. Inhibition of action of acid bacteria and of oxidation times has been shown here. In the case of viabilities, application of the statistical student's "t" test indicates statistically significant differences above the 95% level between the controls and inhibitors on both the Karen and the Robena streams as shown in the tables. Detailed statistical analyses of the other variables of these tests along with those of other tests are given in appendix 1 .

Inhibition in the flooded upward-flowing vertical streams is in contrast with the absence of inhibition in the vertical spray and horizontal systems described below. In consultation with representatives of the sponsors, it was decided to attempt to determine the cause of the difference. One possible cause was felt to be the difference in accessibility of air to the streams since the surface areas of the containers testing Karen and Robena were small relative to those of the other systems. It was decided to aerate the containers for the Karen and Robena tests to see if inhibition may be attenuated. Aeration was initiated on the 116th day after first addition of inhibitor to Robena piles and the 9th day after the addition to Karen piles. Inhibition continued as before but the overall acidity was at a higher level. Analyses were made of dissolved oxygen content of the effluents made three days after aeration of containers 1 through 7 was started. Results are shown in Table 8 below.

TABLE 6 - VIABILITY OF KAREN BACTERIA

Days After Inoculation	#1 No Inhibitor	#2 Raw Inhibitor	#3 Filtrate Inhibitor
	Thousands of Cells per Milliliter		
0	2.4	3.0	28
8	1.4	0.5	0.8
13	3.9	1.2	7
20	14	6.0	8
28	7.6	4.2	0.6
34	8.6	4.4	1.8
44	7.1	3.4	1.1
51	13	1.0	0.5
57	7.5	14.0	0.3
64	3.4	4.5	0.3
71	7.2	0.6	0.3
79	1.6	0.1	0.2
85	1.7	0.1	0
92	1.5	0.4	0.1
99	2.0	12.4	6.4
107	1.6	0.4	6.3
113	2.5	0.7	5.2
120	1.7	1.5	0.7
127	1.0	0.7	1.4
134	0.8	18.8	7.5
142	10	3.2	0.2
150	4.2	< 0.1	0.4
156	1.0	4.5	0.9

Statistical Analysis

	#1	#2*	#3
Sample Size	23	20	22
Mean	4.596	2.49	2.273
Standard Deviation	------	3.00 (1&2)	3.48 (1 & 3)
Student's "t"	------	2.29	2.24
Confidence Level	------	>97.5%	>97.5%

* With two anomalously high values eliminated.

TABLE 7 - VIABILITY OF ROBENA BACTERIA

	Thousands of Cells per Milliliter		
Days After Inoculation	#4 No Inhibitor	#5 Raw Inhibitor	#6 Filtrate Inhibitor
0	3.5	3	1.9
1	25	4.1	2.0
4	4	0.2	0.8
15	3	1.7	0.2
22	3.9	2.0	1.5
30	1	0.8	0.2
35	1.8	0.4	0.5
42	3	1.7	0.6
40	2.8	0.6	0.3
56	3.6	0.9	0.4
66	4.0	0.3	0.2
73	3.5	0.8	0.3
79	1.8	0.2	0.1
86	3.2	1.1	0.6
93	1.7	0.4	0.2
101	1.2	0.1	0
107	0.3	0	0
114	0.4	0.2	<0.1
121	2.0	0.2	0.1
129	1.1	<0.1	<0.1
135	0.1	0.2	0.1
142	1.2	0.1	0.2
149	0.5	0.1	11.5
156	0.8	<0.1	<0.1
164	0.8	0.2	0.2
172	0.8	0.7	<0.1
178	0.1	<0.1	1.8

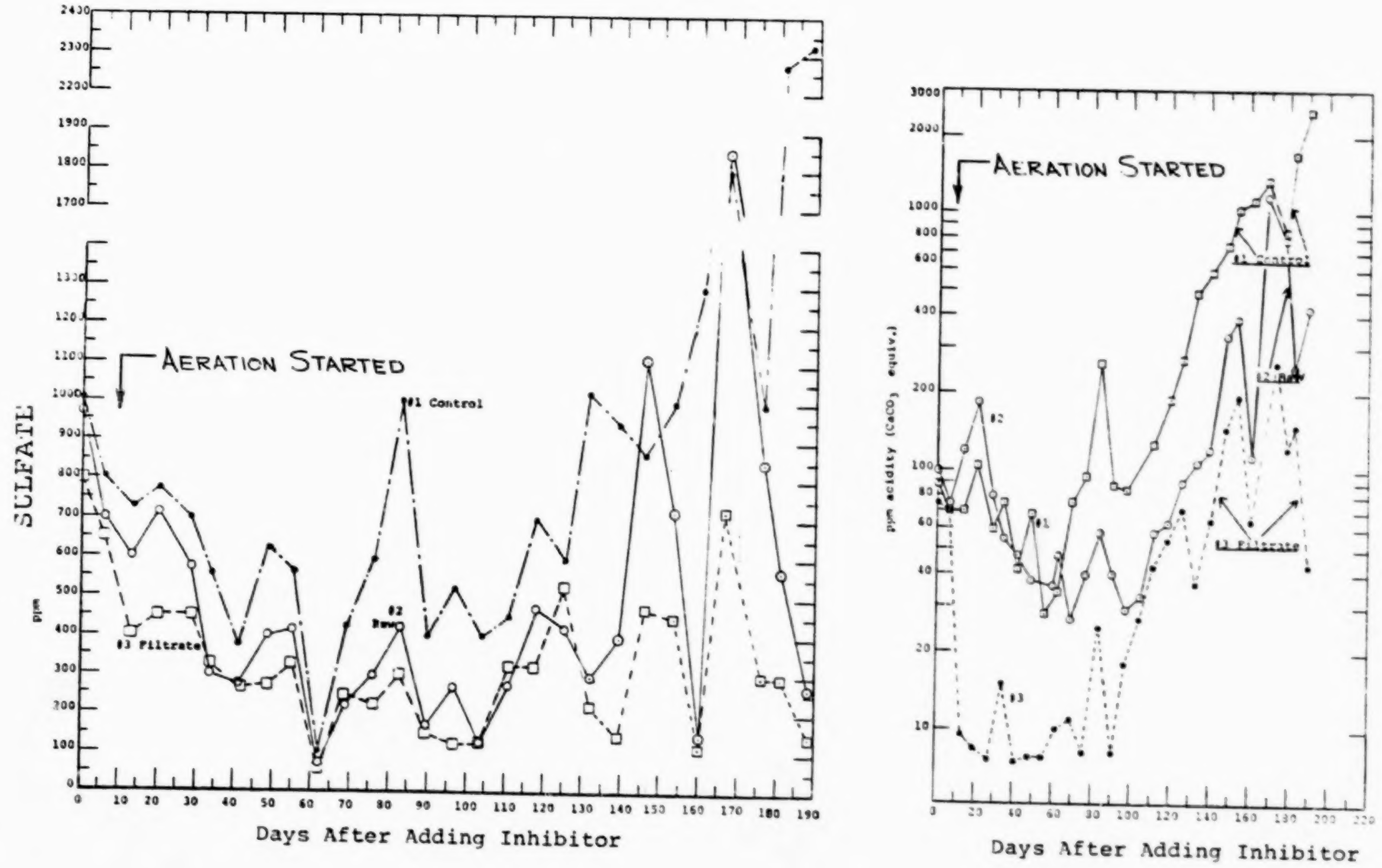


Figure 2 - Effect of Inhibitor Upon Production of Sulfate and Acid by Karen Bacteria

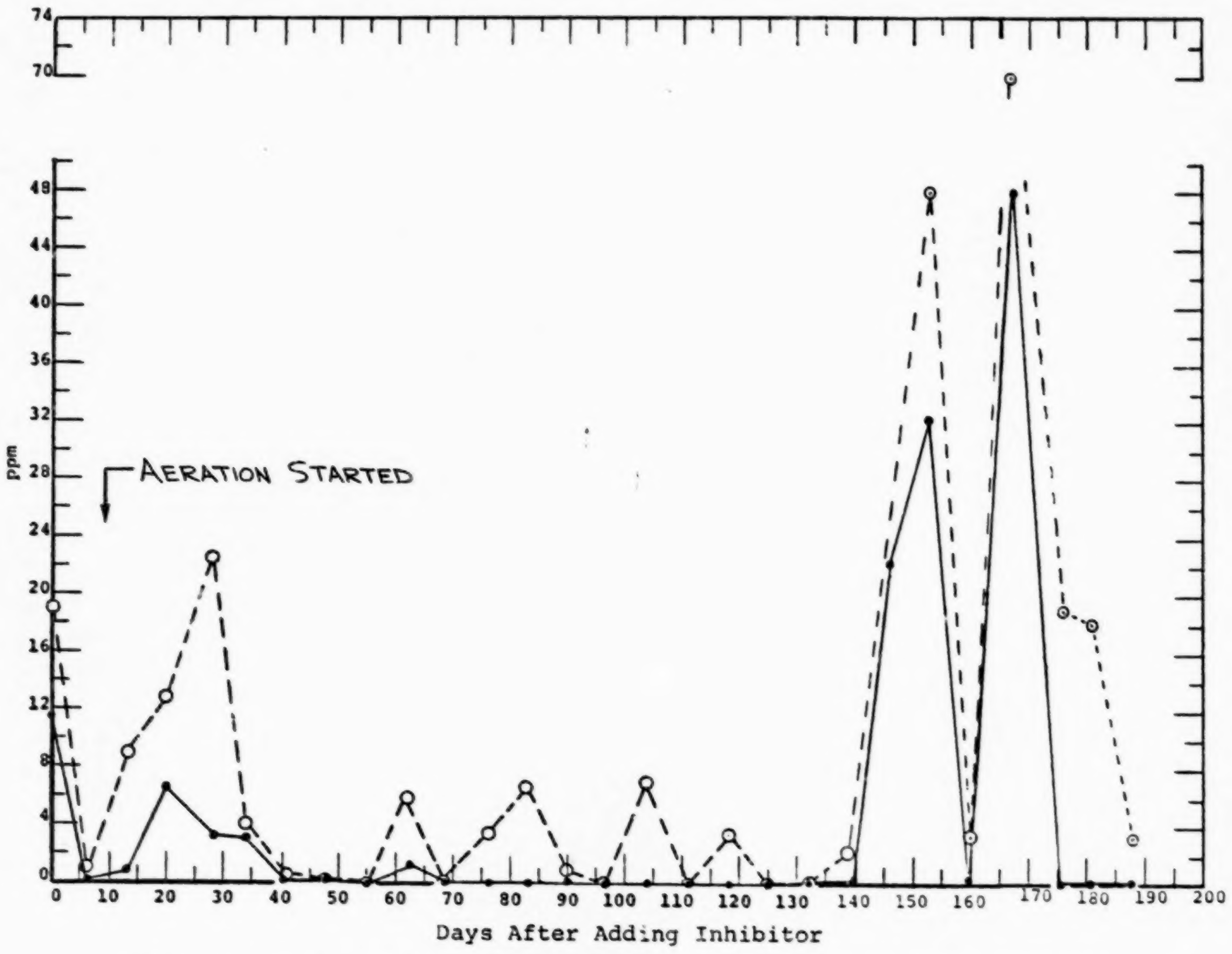


Figure 3 - Ferrous (_____) and Total (-----) Iron by Karen Bacteria with Filtrate Inhibitor

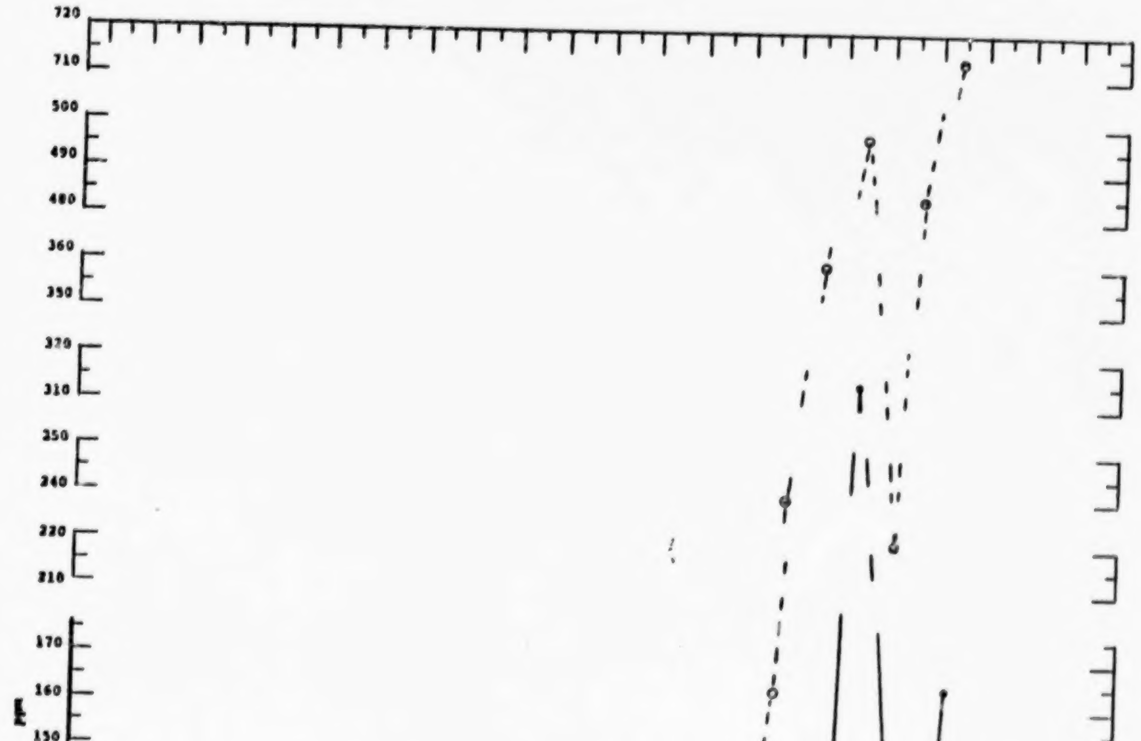


Figure 4 - Ferrous (____) and Total (----) Iron Production by Uninhibited Karen Bacteria

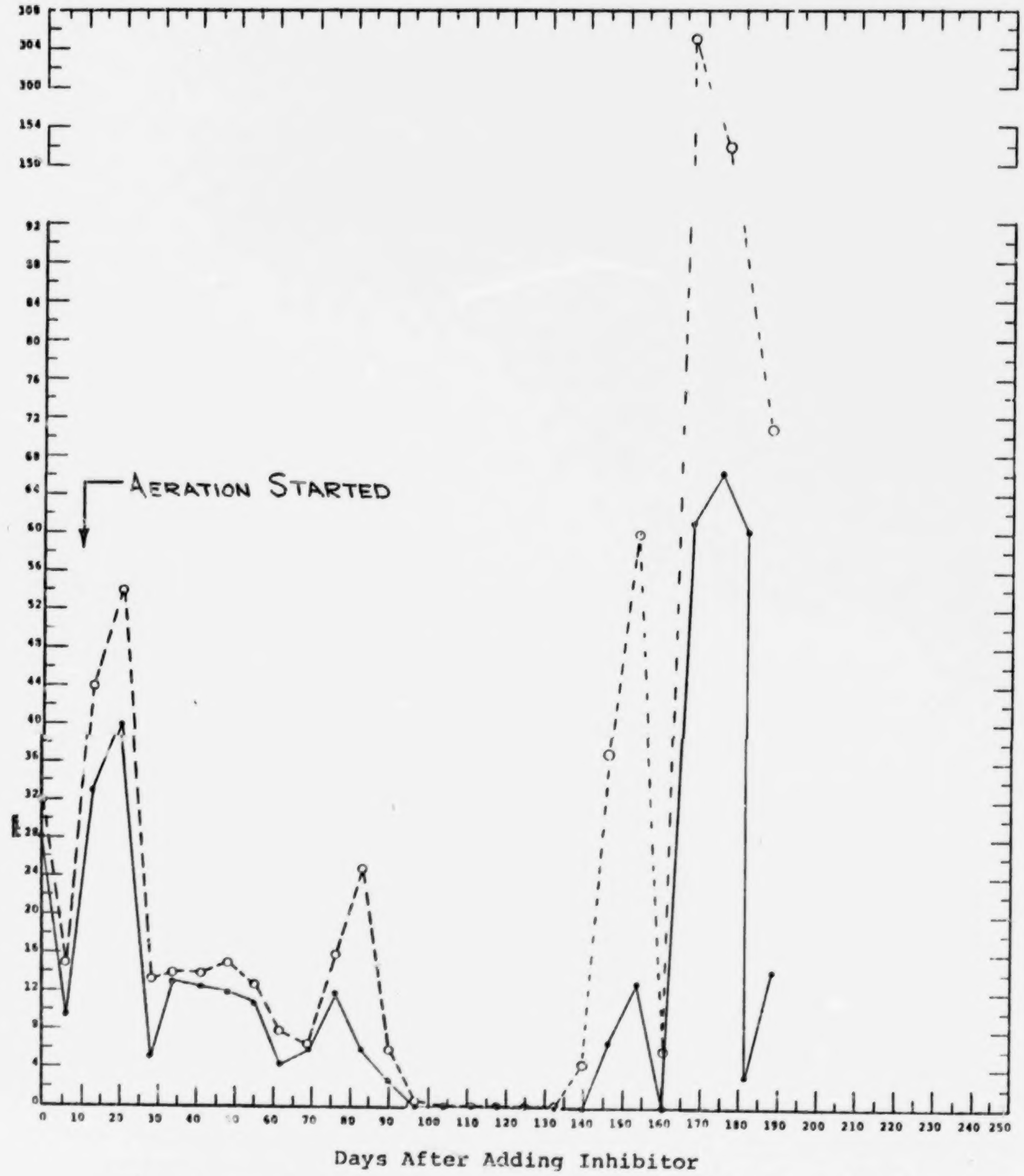


Figure 5 - Ferrous (____) and Total Iron (----) Production by Karen Bacteria with Raw Inhibitor

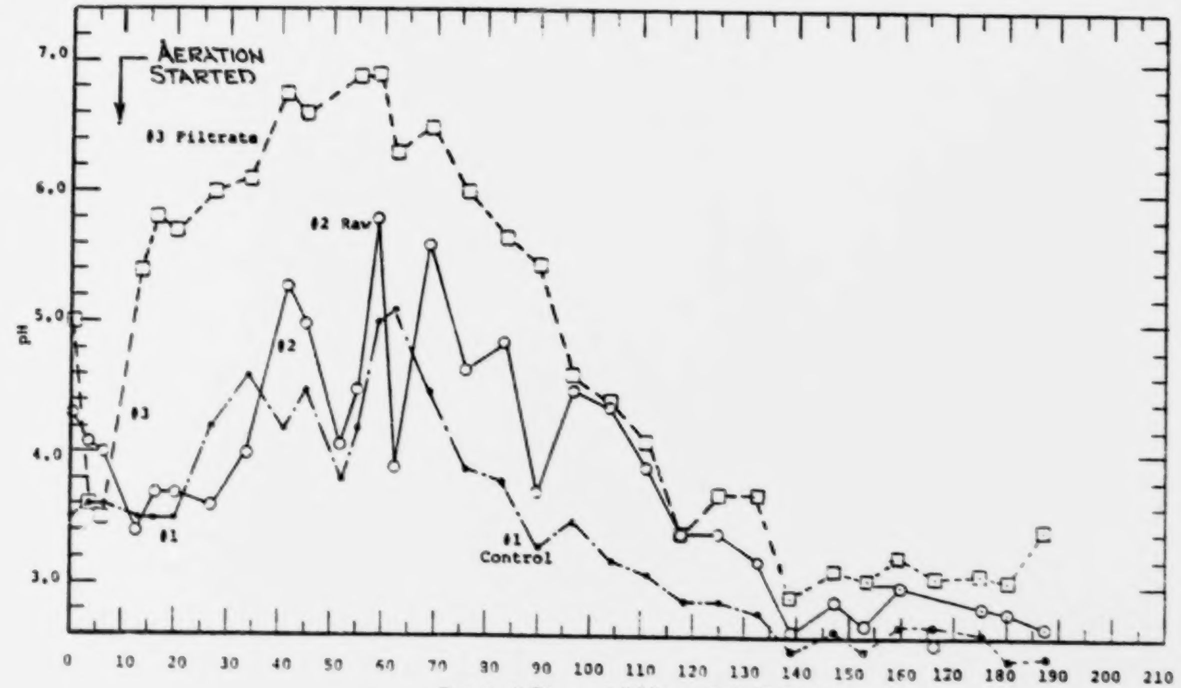


Figure 6 - Effect of Inhibitor Upon pH in Karen Cultures

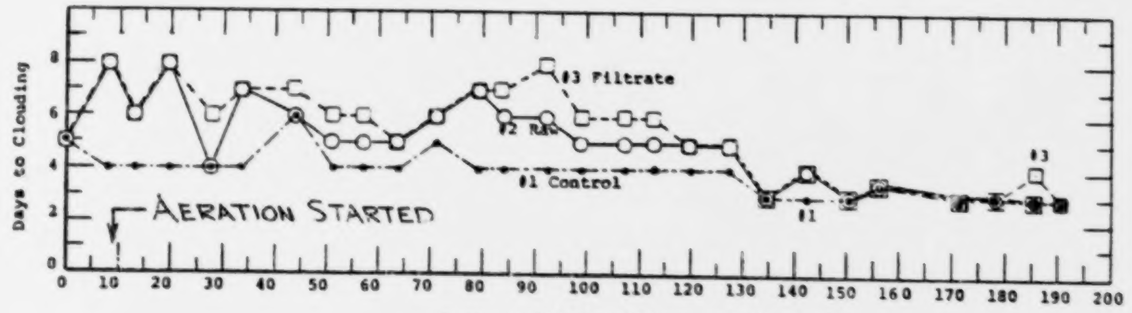


Figure 7 - Oxidation Times for Karen Bacteria

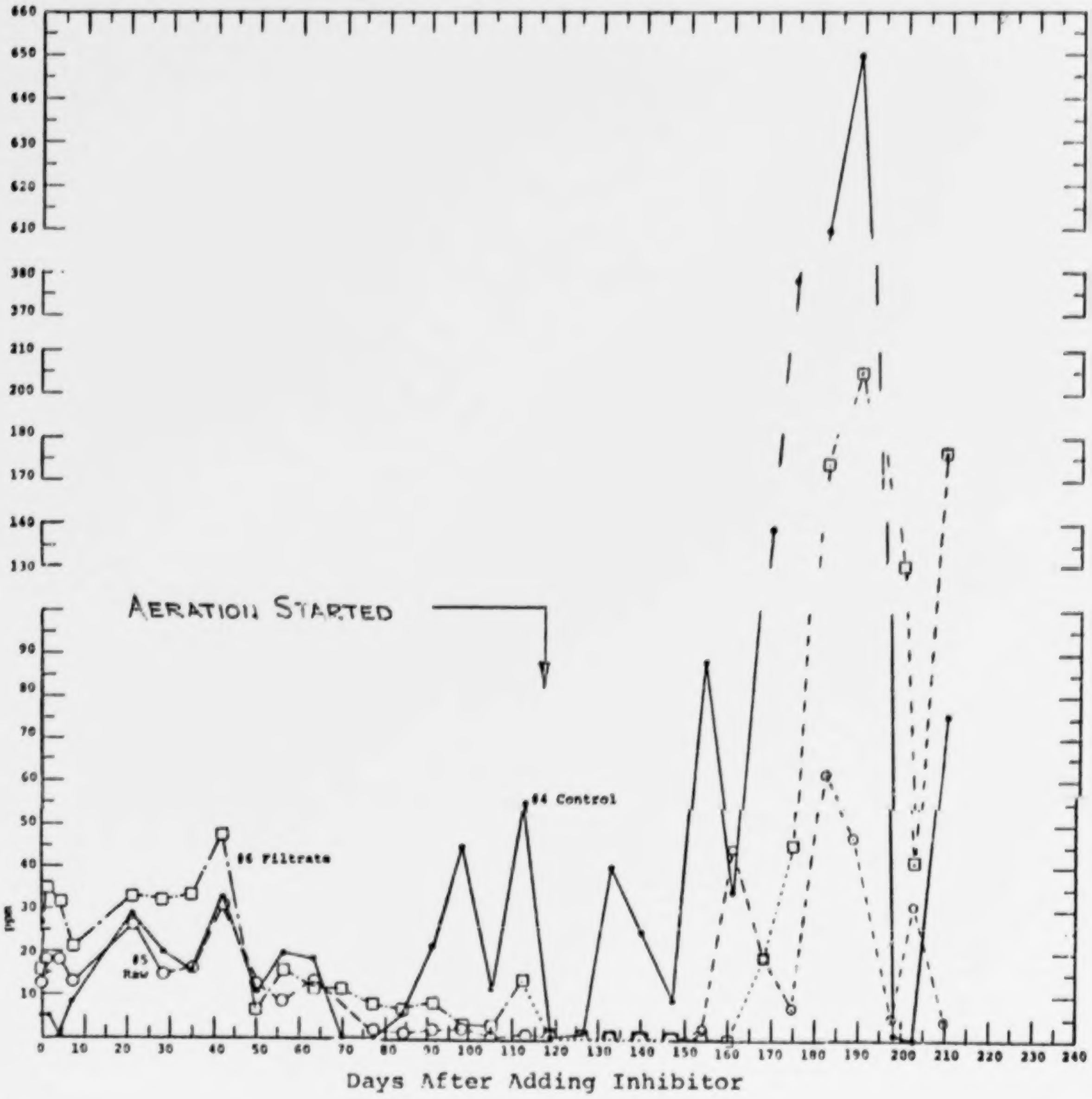


Figure 8 - Inhibitors for Ferrous Iron Production by Robena Bacteria

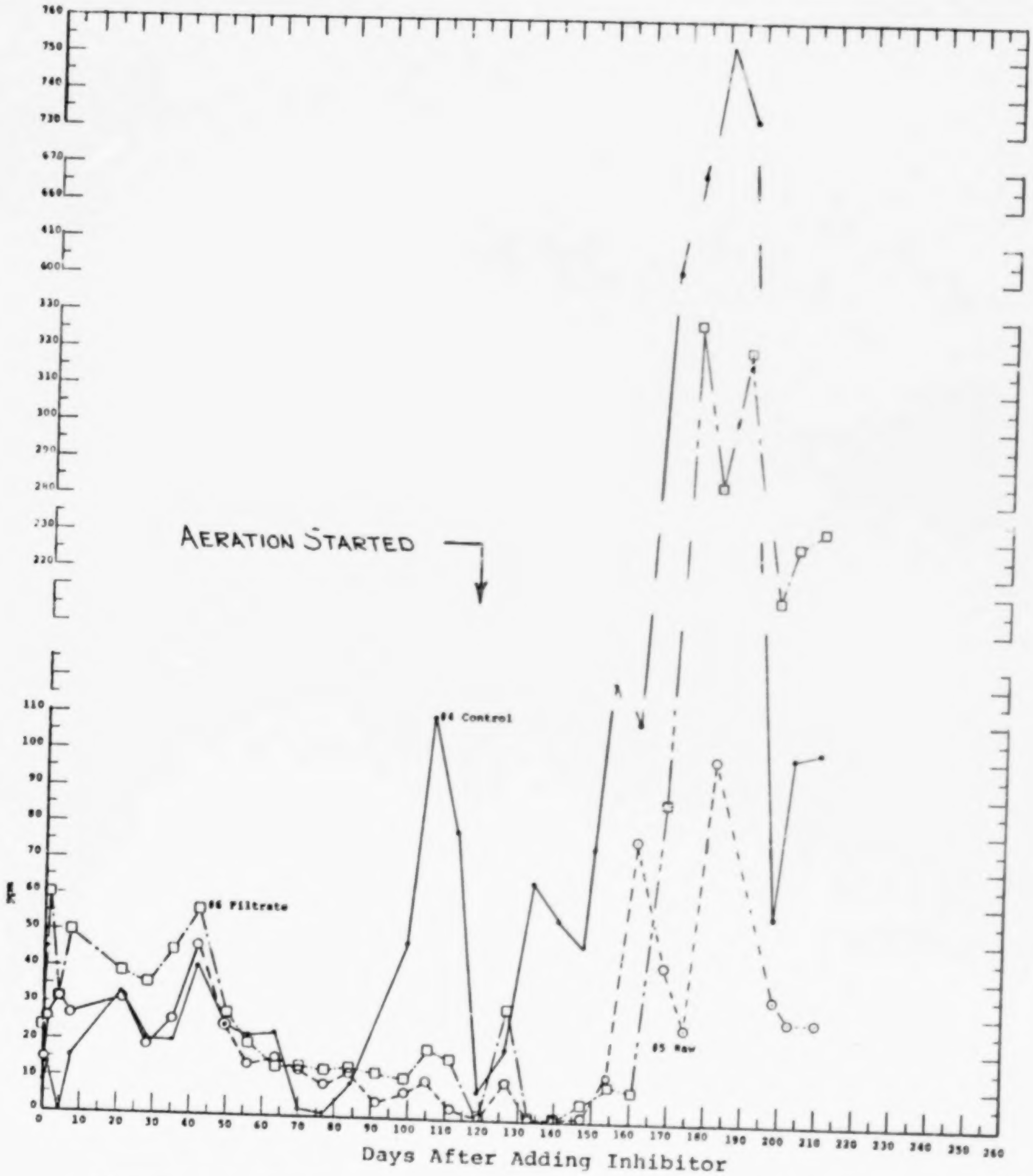


Figure 9 - Effect of Inhibitor on Production of Total Iron in Robena Cultures

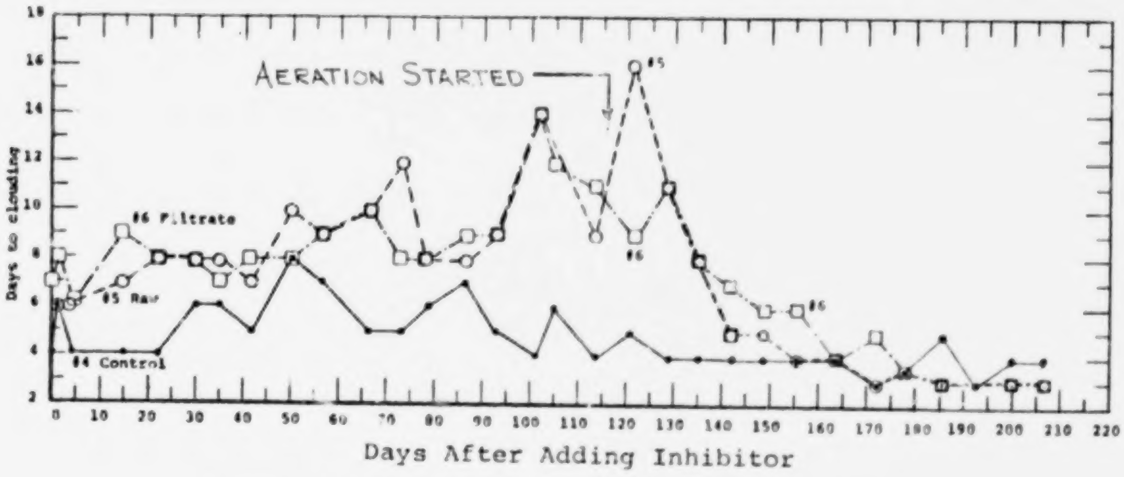


Figure 10 - Oxidation Times of Robena Bacteria

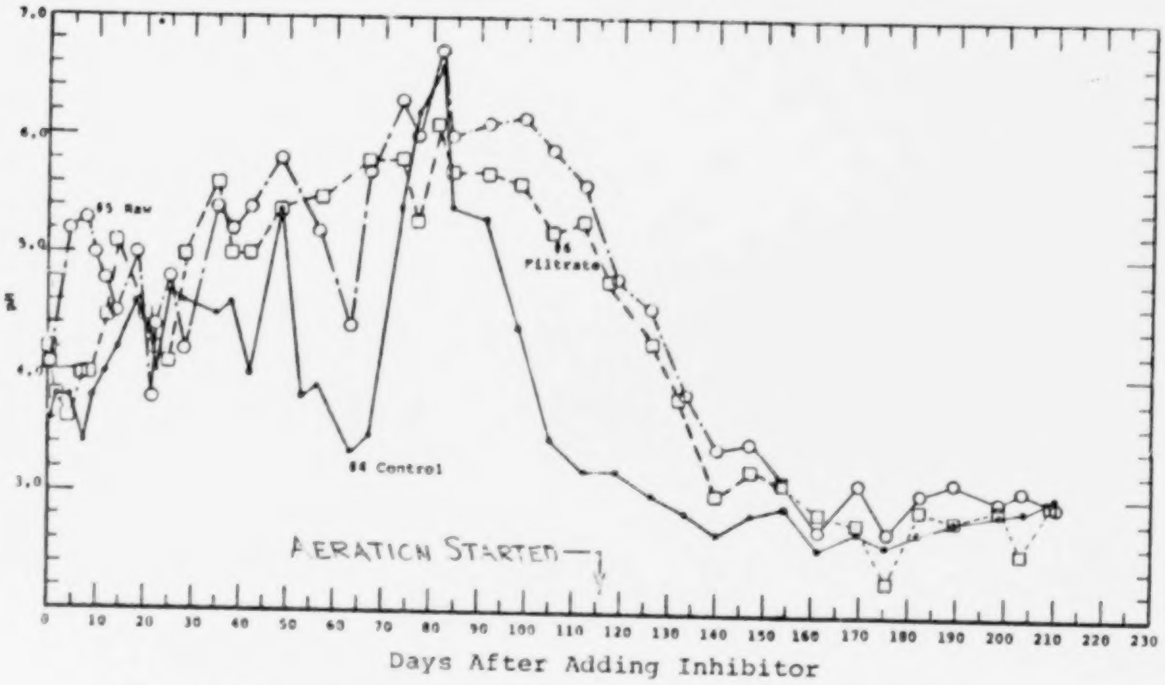


Figure 11 - Effect of Inhibitor on pH of Robena Cultures

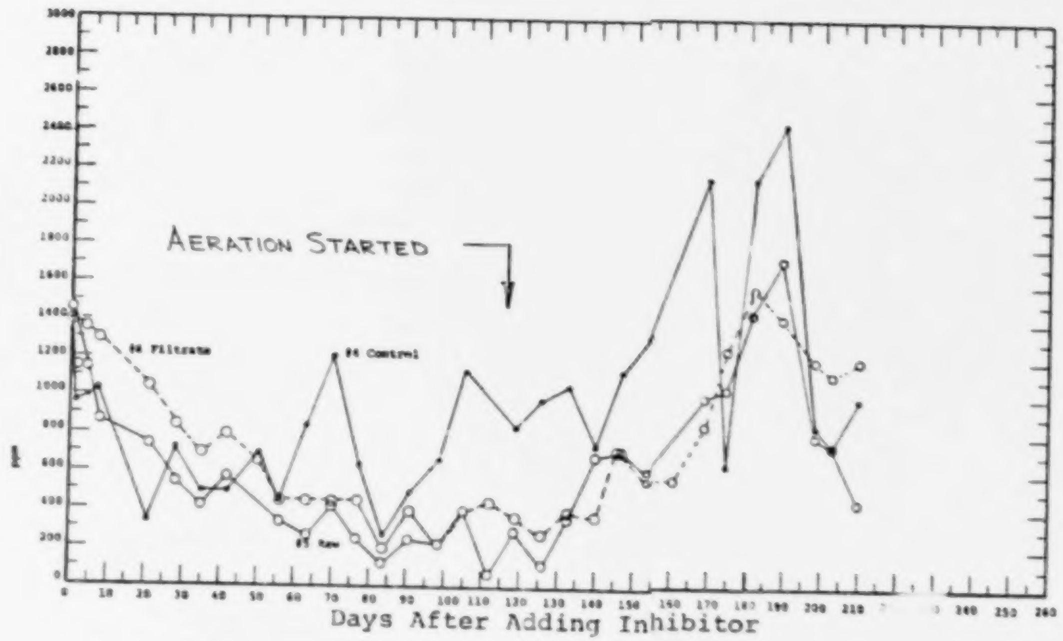


Figure 12 - Effect of Inhibitor on Sulfate Production by Robena Bacteria

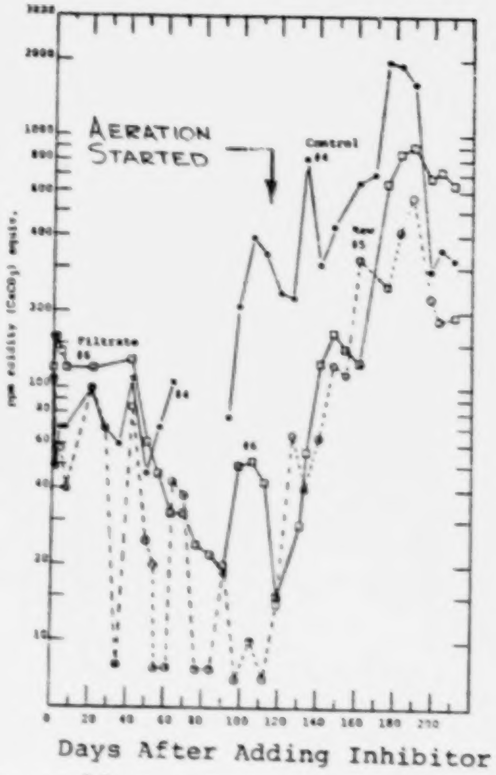


Figure 13 - Effects of Inhibitors on Acid Production by Robena Bacteria

TABLE 8 - DISSOLVED OXYGEN CONTENT OF VARIOUS STREAMS

Culture No.	1	2	3	4	5	6	7
ppm	2.41	3.18	2.46	2.25	1.65	2.00	1.78
Influent water	1.94						

There is wide variability in oxygen contents with no consistent patters from which conclusions can be drawn except that aeration is inefficient.

Horizontal System

In order to determine if unconcentrated natural inhibitor might be effective when introduced in the influent of flooded downward flowing horizontal systems cultures of bacteria from an effluent from Robena mine were introduced initially into three of the horizontal troughs. The experimental setup is shown in Figure 14. A water flow was established at the rate of 15 ml/min. When the bacterial population was established as determined by chemical and bacterial analysis of the effluent a liter of raw natural mine drainage supposedly containing the inhibitor was introduced into trough #1 at the inlet. A second liter was introduced after 34 days. Filtrate inhibitor (100 ml) was introduced into trough 1 on the 37th day and another liter in the 63rd day. To see if more abundant quantities of natural inhibitor might be effective along the horizontal system, three liters of inhibitor were added to one of the troughs. Results are given in Appendix 2. Here the first identifying digit represents the trough number and the second the sample port number, starting with the influent end. Thus 1-2 represents sample port #2 in trough #1. Some data points are lacking because mechanical difficulties prevented sampling troughs. No inhibition was noted from the unconcentrated unoptimized raw mine drainage downstream along the horizontal system.

Dissolved oxygen was determined at various sample ports along the horizontal systems on the 106th day after first addition of inhibitor to one of them. Results are shown in Table 9.

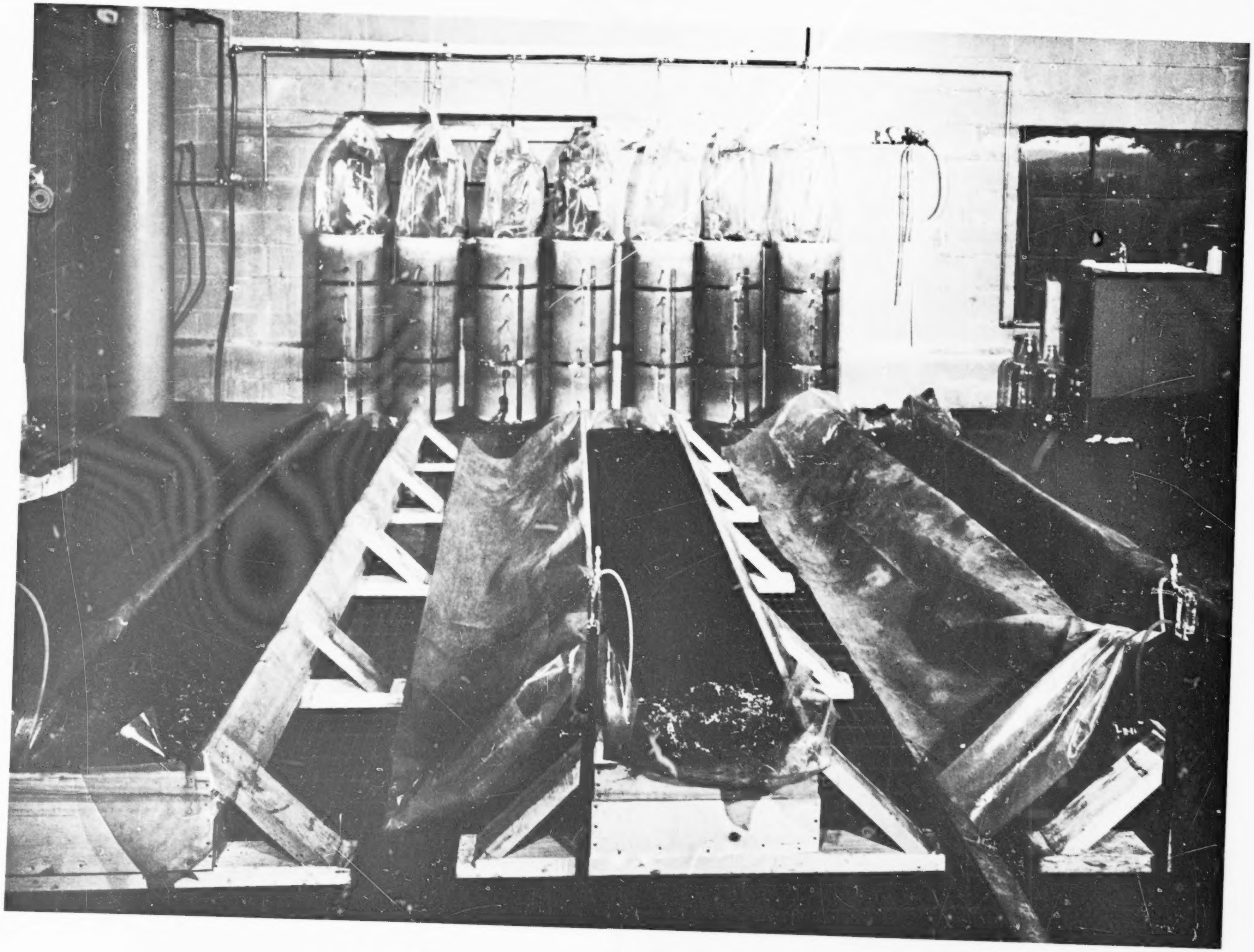


FIGURE 14 FLOODED HORIZONTALLY FLOWING SYSTEMS

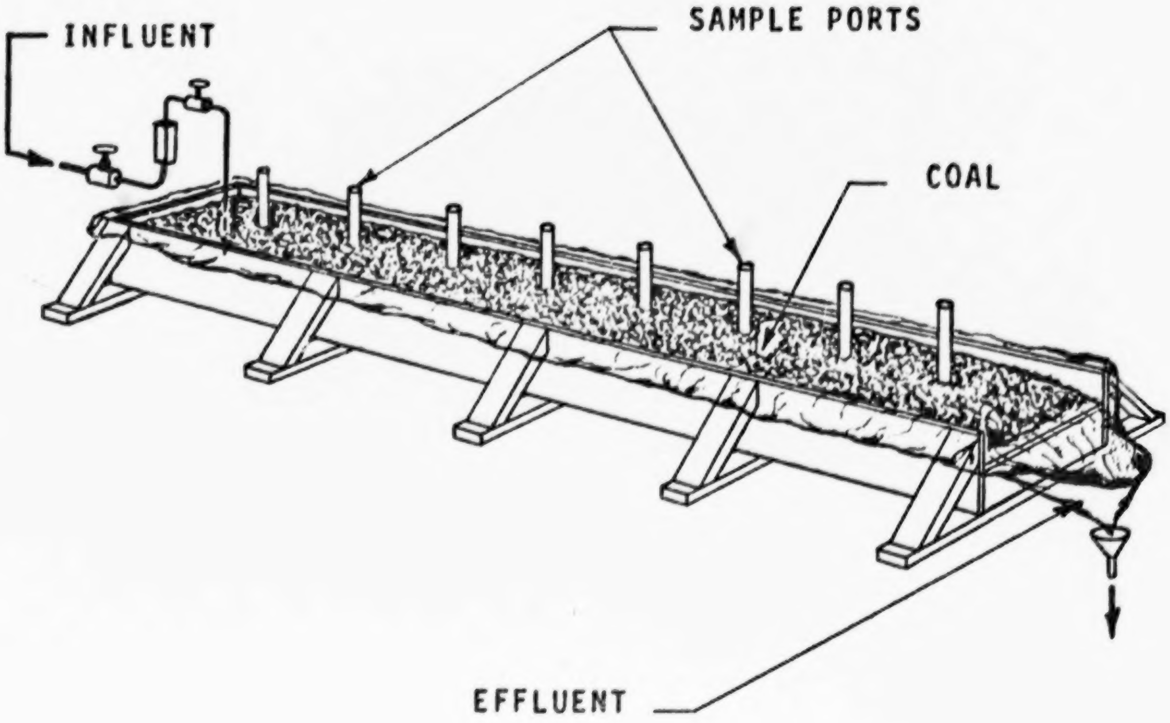


FIGURE 14a FLOODED HORIZONTALLY FLOWING SYSTEM-DETAIL

TABLE 9 - DISSOLVED OXYGEN (ppm) AT VARIOUS PORTS IN HORIZONTAL SYSTEMS

Sample Port Numbers	1	2	4	5	9
Trough 1 (with inhibitor)	1.76	1.41	3.68	4.32	8.89
Trough 2 (no inhibitor)	1.59	1.71	2.92	2.86	6.48

The dissolved oxygen contents of these systems were significantly greater at the effluent end than those of the laboratory coal piles used to study inhibition of Karen and Robena bacteria. At the influent end values are close to that of the tap water (about 1.9 ppm). It would appear that at the upper end of the pile the oxygen is being consumed in the pyrite oxidation or the stream is being aerated.

Unflooded Downward-Flowing Vertical System

Acid is often produced in mines where water percolation drips from the roof into pools in the floor. In order to determine if the natural inhibitory waters studied in other systems could be effective when introduced into pools at the bottom, the unflooded downward-flowing vertical systems described in Section 7 were put into operation. Cultures of bacteria were added as follows: Ferrobacillus sulfooxidans to columns #1 and #2, Ferrobacillus ferrooxidans to #3 and #4, a mixture of the two species to #5 and #6; and #7 remained sterile. The setup is shown in Figure 15. After the bacteria had been well established as evidenced by chemical and bacterial analyses showing increased acidity and high viabilities in effluents, inhibitors were added to the pool at the bottom of the column in accordance with Table 10. Coal piles without added inhibitors were maintained as controls.

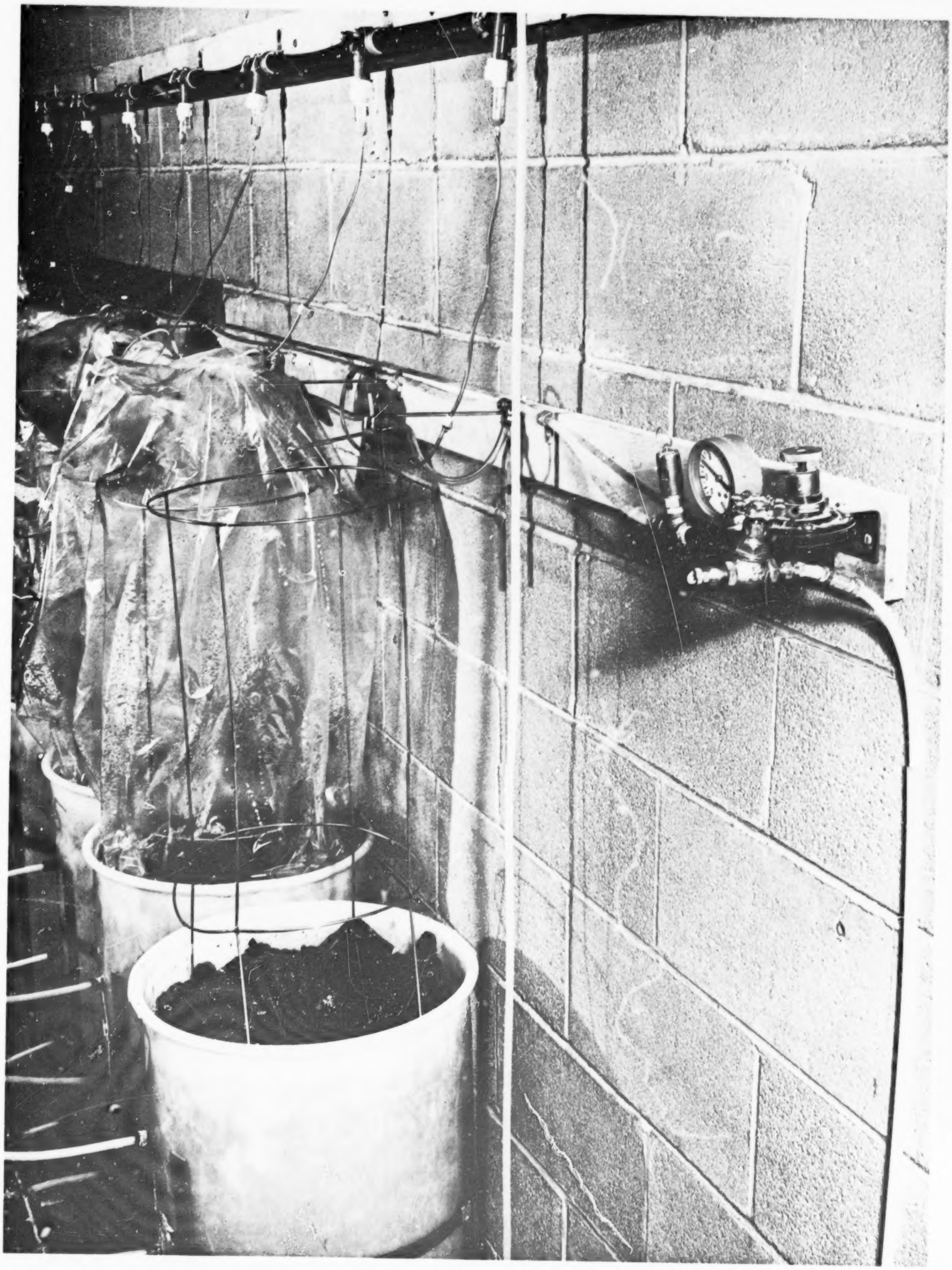


FIGURE 15 VERTICAL SPRAY SYSTEMS - NOT FLOODED

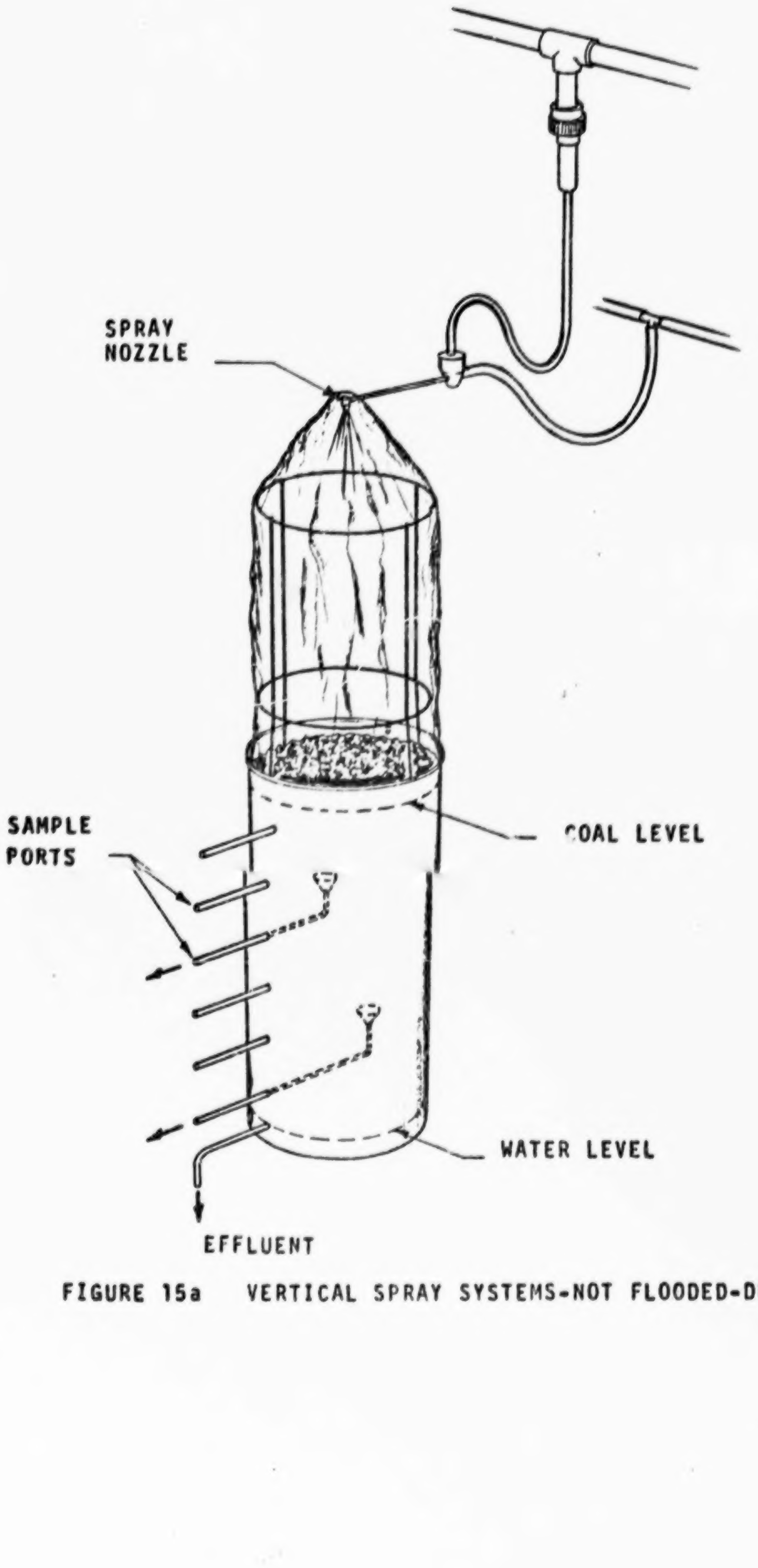


FIGURE 15a VERTICAL SPRAY SYSTEMS-NOT FLOODED-DETAIL

TABLE 10 - ADDITION OF INHIBITORS TO VERTICAL SPRAY SYSTEMS

Cultures	Amount & Type of Inhibitor	Days After First Addition
#2 F. Sulfo	300 ml raw Wildwood	0
F. Sulfo	800 ml Wildwood filtrate	48
#4 F. Ferro	300 ml raw McCormick	0
F. Ferro	800 ml McCormick Filtrate	48
#5 Mixed	150 ml each of 2 above raw sources	0
	400 ml each of 2 above filtrates	48

No evidence of inhibition by unconcentrated unoptimized natural inhibitors was shown in any of the cultures. Data are given in tables in Appendix 2. Throughout its first digit represents the number of the system and the second digit the number of the sample port stating at the top (1-1, 2-1, 3-1, etc) and ending with the pool at the bottom (1-7, 2-7, 3-7, etc). Acidities were determined only on samples from the bottom port because of the large size of the sample required for titration. Data on tank #7 are not given after the 79th day because it was flooded after that time. Some data points are lacking because mechanical difficulties prevented sampling. It was decided to modify one of the containers to simulate the containers of Robena and Karen cultures to see if inhibition would result. This was done by sealing off the bottom sample port drain and using the top sample port as the effluent. Filtered tap water was introduced into the bottom. Total acidity fell from the range of 200-300 ppm to 20-30 ppm as a result of this action, presumably because of lessened access of air to the system. Other chemical properties are similar to those of the systems for Karen and Robena bacteria.

SECTION 10

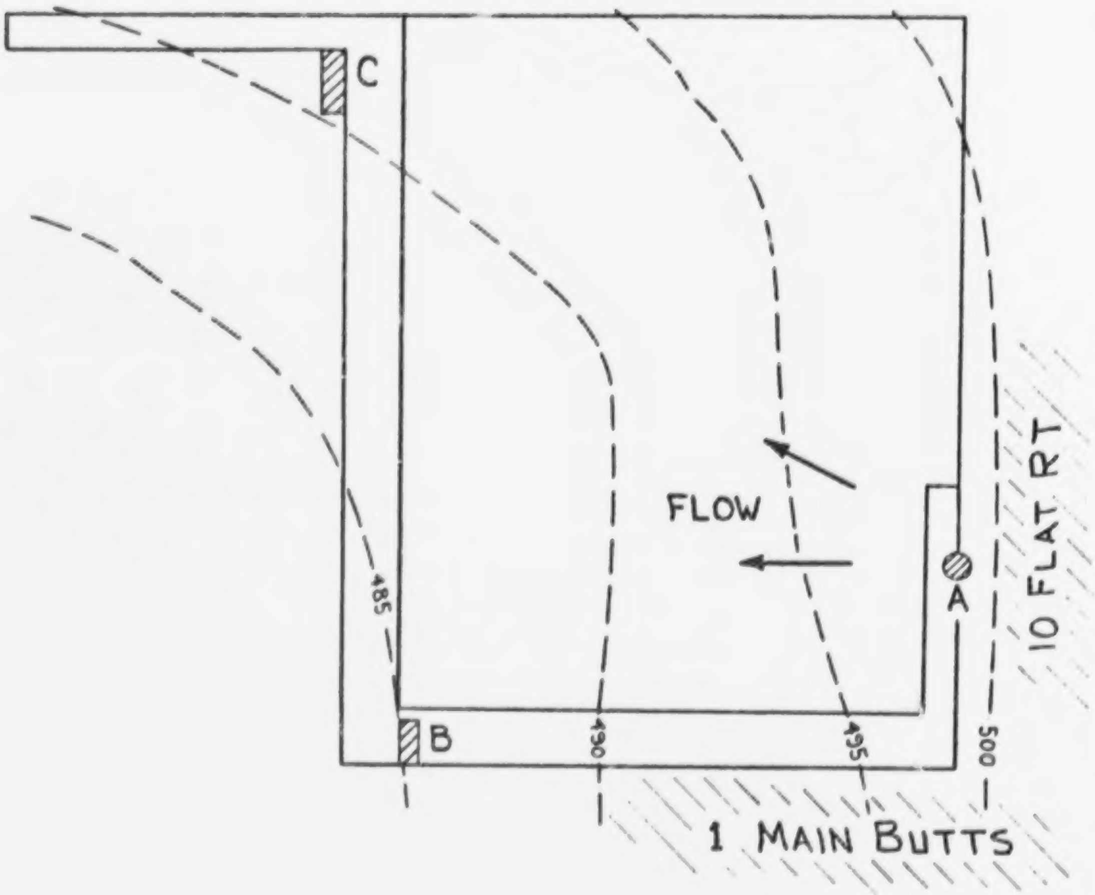
FIELD TESTS IN ROBENA MINE WITH NATURAL INHIBITORS

The mined-out area on the left side of 1 Main-10 Right, United States Steel Company's Robena No. 2 Mine was chosen for the test site. Mine water from this area flows naturally to the two (2) pump pickup locations shown in Figure 16. The pH of the mine water is normally about 3.0. The flow rate is normally considerably less than 300 gpm, perhaps even less than 100 gpm. The coal contours are such that water containing the inhibitor introduced in the abandoned 10 Right Flat should flow thorugh the area to the 11 Right and 12 Right pump pickups and, therefore, come in contact with the mine water somewhere in the region.

Before attempting to introduce water containing inhibitor into the system, water from the mine fresh water system was directed to the area with Calcocid Blue 2 G (Pylam Products Company) dye to determine if it would report to the two (2) pump pickup locations. Table 11 gives quantities introduced and treatment of water used. The dye did not show up at the pump pickups possibly because of adsorption by the gob; however, an increase in the pH (presumably from dilution) from 2.7 to as high as 4.1 at the 11 Right and from 3.1 to as high as 4.2 at the 12 Right locations indicated that the water might have traversed this area. See Tables 12 and 13.

Water from the region of Wildwood mine was injected on February 24. It had a pH of 4.5 and contained 37 ppm total acidity, 150 ppm sulfate, 2800 ppm undissolved suspended solids and 1 ppm total iron. This was hauled to Robena's Hartley Shaft in three (3) 4,500-gallon tank trucks from Wildwood Mine. The 5-inch fresh water line from Hartley Shaft bottom to 1 Main-10 Right was separated from the fresh water system and connected to a fire hydrant at the surface. The 13,500 gallons were dumped into the hydrant and drained via the 5-inch line into the 10 Right test area.

Two (2) samples were taken once each week at the 11 Right and 12 Right locations. Results are shown in Tables 12 and 13. No inhibition was noted possibly because topography prevented access of inhibitor to some acid-producing regions. Furthermore, because of variability in the natural inhibitor, inhibitory power was absent at the time the water was collected as evidenced later in laboratory tests, which showed no inhibition in liquid cultures of iron bacteria. Laboratory tests made in July 1969 showed that some weak inhibitory power had returned;



LEGEND

A - Trace water and inhibitor introduced at this location.
B - 11 right pump pickup.
C - 12 right pump pickup.

FIGURE 16 - LOCATIONS OF INHIBITOR INJECTION POINT AND DRAINAGE PICKUP POINTS IN ROBENA MINE

TABLE 11 - VOLUMES OF TRACE WATER AND INHIBITOR INTRODUCED INTO ROBENA MINE

Date	Gallons	Dye Added
November 21, 1968	12,000	Yes
January 8, 1969	13,500	Yes
February 3, 1969	12,000	Yes
February 5,6,7, 1969	102,000*	No
February 7, 1969	15,500	Yes
February 10, 1969	14,500	Yes
February 11, 1969	7,500	Yes
February 24, 1969	13,500	Water from Wildwood Mine Containing Inhibitor
July 24, 1969	13,000	"

* This water added untreated to fill the local depressions in the gob.

TABLE 12 - ANALYSES OF WATER FROM - 11 RIGHT

Date Sampled	pH	Total Acidity	Total Iron	SO_3
1/8/69	2.7	2720	493.0	5224
1/9/69	2.8	2720	504.0	5267
1/10/69	2.8	2840	526.0	4821
1/11/69	2.8	2910	538.0	4763
1/12/69	2.7	2880	543.0	4857
2/8/69	3.3	1800	375.0	4977
2/9/69	4.1	1000	336.0	4909
2/10/69	3.8	2150	717.0	4958
2/11/69	3.5	1600	428.0	4949
2/12/69	3.8	2750	818.0	4967
2/13/69	3.5	3550	582.0	5045
2/14/69	3.3	4250	459.0	4893
2/24/69*	3.0	3100	481.0	4767
3/3/69	4.4	3800	1075.0	5063
3/10/69	4.6	3550	620.0	3851
3/17.69	3.8	3400	810.0	4405
3/24/69	4.3	3500	750	4334
4/7/69	3.8	3530	870	4259
4/14/69	3.9	3600	710	4366
4/21/69	3.5	2950	560	4337
4/28/69	3.4	4700	1000	5315
7/3/69	3.5	2240	655	----
8/4/69	3.45	3230	----	----

* -Inhibitor added. See Table 11.

TABLE 13 - ANALYSES OF WATER FROM - 12 RIGHT

Date Sampled	pH	Total Acidity	Total Iron	SO_3
1/8/69	2.9	3110	1008.0	5228
1/9/69	3.1	3200	1002.0	4899
1/10/69	3.0	3180	1081.0	4809
1/11/69	3.1	3240	1008.0	4811
1/12/69	3.1	3400	1109.0	4755
2/8/69	3.0	3080	937.0	3939
2/9/69	3.3	3100	922.0	4230
2/10/69	3.2	3100	1002.0	4910
2/11/69	3.2	4200	1142.0	5009
2/12/69	3.5	4350	1165.0	4943
2/13/69	3.7	5400	1154.0	4990
2/14/69	3.4	5824	1165.0	4653
2/24/69*	4.2	5400	974.0	4586
3/3/69	3.4	6150	1198.0	4936
3/10/69	3.9	5550	820.0	4018
3/17/69	3.1	4800	955.0	4590
3/24/69	3.5	5250	925.0	4568
4/7/69	2.8	6200	1150.0	4322
4/14/69	2.9	4900	880.0	4847
4/21/69	2.9	5000	855.0	4384
4/28/69	3.4	5450	955.0	4791
7/3/69	2.9	3420	795.0	----
8/4/69	2.8	3390	----	----

* - Inhibitor added. See Table 11.

and another field test at Robena was initiated in that month. Progress will be monitored using company funds after completion of the contract.

SECTION 11

ATTEMPTS TO ISOLATE, CONCENTRATE AND IDENTIFY PHAGES

Background

It seems in order to review some of the previous work done on attempts to isolate phage specific for the iron bacteria and to review the criteria for proof of existence of phage. Our previous work had not given definite proof that iron bacteria inhibition was caused by the existence of phage. However, there were indications that some type of biological agent was responsible for inhibition of iron bacteria. Previous experimental work can be found as given by Shearer and Everson, (1967). Briefly, experimental results showed that certain species of iron bacteria were lysed or inhibited by Wildwood and McCormicK filtrates on agar plates. This inhibitory material could be transferred four or five times without losing its potency. It is doubtful that a chemical inhibiting agent could retain its potency through transfers, particularly as each plate transferred extract was filtered through a 0.20 micron filter. In addition, each filtrate was specific for the strain of iron bacteria against which it was prepared. Electron microscopy showed unidentified particles associated with lysis of iron bacteria; however, no photographs of a phage of known structure were obtained.

Prior to work on the present contract our proposal stated our attempts to identify phage had been unsuccessful, but work on phage would be continued along with that on other natural biological agents which might prove to be inhibitory to iron bacteria. This includes competition from other species of bacteria, specifically Caulobacter (previously found in filtrates by electron microscopy). The Caulobacter may also parasitize iron bacteria. Still other modes of inhibition may be through the action of antibiotics, enzymes and bacteriocins. Attempts to find phage (the only final proof being the demonstration of phage in electron micrographs) were continued in our present study. Attempts were also made to determine if viruses closely related to phage were present. A discussion of this will be included in the following section on phage, leaving work on Caulobacter and antibiotics as separate studies included later on in this report.

Phage - Technical Methods of Study

The specific test iron bacteria were isolated from their sources of acid mine water and grown in nutrient synthetic I acid mine drainage required for their maintenance and propagation. Additional strains of autotrophic iron and sulfur bacteria were obtained from the American Type Culture collection. The stock strains obtained were *F. ferrooxidans*, and *F. sulfooxidans*.

A search for phage required that potential phage material be tested against the 4 strains of iron bacteria, the 2 stock cultures, *F. sulfo* isolated from Robena test site and the *F. sulfo* isolated from Karen test site. It was necessary to make frequent transfers in order to keep these iron microorganisms viable.

Viral phages (bacteriophages) were sought in the natural habitat of the host bacteria, i.e., soil, mud, mine drainage, coal stockpiles and refuse dumps. The viable iron bacteria were incubated in synthetic I mine drainage, then inoculated with the potential phage source liquids in equal quantities. After a second incubation period, the cultures were centrifuged to partially remove bacteria and the supernates filtered through a 0.20 micron millipore filter to remove bacteria. The filtrates are tested for the presence of phage by:

a. Plating - Filtrate aliquots were diluted serially by synthetic mine drainage and added in varying amounts to each well-oxidized iron bacterium already growing in synthetic mine drainage. Aliquots of this mixture were then added to soft agar and the contents added to iron agar plates by pouring over the surface of solid agar and rotating. After solidifying, the iron agar plates were incubated and the plates examined for plaques of lysis in the film of iron bacteria growth.

b. Lysis of liquid culture - The synthetic I iron solutions were inoculated with actively growing cultures of the various strains of iron bacteria and filtrates. After incubation, the tubes were examined for bacterial oxidation of ferrous iron and compared with a bacterial control tube inoculated with iron bacteria but not with filtrate. Phage or a lytic agent is indicated indirectly when iron oxidation does not occur, or when tube contents are partially oxidized. Suspected phages were then further tested for by plating aliquots from non-oxidized tubes as in (a). If phage was obtained on an iron agar plate, it was picked or eluted, millipore filtered, serially diluted and tested against fresh iron bacteria on plates.

Inhibition is often found on plates but not in liquids and the reverse is also true. Adams, p-448 (1959) has observed that a phage which brings about complete lysis of both cultures of bacteria may produce very tiny plaques, may have a low efficiency of plating or a slow adsorption rate or may be otherwise unsuitbale for quantitative work by plaque-counting techniques. Also, a phage which produces beautiful plaques may not bring about lysis in broth cultures, may yield only low titer stock or be difficult to work with in other ways. The method of isolation may in part determine the properties of the phage isolated, through isolation of variants.

Potential Phage Source Testing of Test Site Drainages

Ten neutral or alkaline drainages from the Robena and Maple Creek complexes were prepared as bacteria-free filtrates and tested on solid and liquid iron media. The filtrates were prepared by inoculating each drainage into synthetic iron cultures of each strain of bacteria, *F. ferro* (stock culture), Karen mine-isolated iron bacteria (*F. sulfo*), and Robena mine-isolated iron bacteria (*F. sulfo*). After incubation, each drainage/bacterial culture was centrifuged and millipore-filtered to obtain the bacteria-free filtrate. This is one method for detecting phage according to Adams, (1959). Each filtrate was then tested against its specific strain of bacteria on solid agar plates and in liquid synthetic iron media. The tubes and plates were then incubated and observed for ferrous iron oxidation in liquid media and for appearance of iron bacteria colonies on plates. Positive bacterial controls were included in which bacteria were added to the tubes and plates but no potential inhibitor. Tube and plate tests were always compared with the positive bacterial controls. Negative bacterial control tubes were also included in which neither bacteria or filtrate were added to a tube of synthetic acid mine drainage.

None of the ten neutral or alkaline drainages contained an inhibitor against the iron bacteria.

Attempts to Isolate Phage from Other Mine Areas

Five potential phage sources from J & L mine were also tested against the four strains of bacteria as previously described. These sources were untreated drainages of neutral pH. The prepared filtrates did not prevent ferrous iron oxidation by each of the four bacterial strains in liquid media. Slight bacterial reduction and plaques were seen on plates containing Karen bacteria and four J & L filtrates. It was evident that four of the filtrates showed some slight inhibitory action on Karen bacteria. The five filtrates were not effective when tested on solid media against the other three strains of bacteria.

A neutral drainage from the McCormick mine areas was prepared as a filtrate and tested against the four strains of bacteria. Ferrous iron oxidation was prevented in liquid media for two days beyond the control culture of stock F. ferro. Plaques, but no reduction in density of growth in non-plaque areas, appeared on plates of F. ferro and filtrate. However, results of testing the McCormick source against the other three strains of iron bacteria were negative in that no bacterial inhibition occurred on solid or liquid media. This is shown on Table 14.

Three Wildwood mine waters, neutral or slightly acidic, were collected from various points within the mine area. These water sources were also prepared as bacteria-free filtrates against three strains of iron bacteria, F. sulfo, Karen and Robena. Plates of iron bacteria isolated from the Karen and Robena mines were partially clear of bacteria at 1:5 and 1:10 filtrate to bacteria ratios. The plates at a 1:100 and 1:1000 dilution ratios showed little clearing, but many plaque-like areas in the growth of Karen iron bacteria. Plaque-like areas were also seen at 1:100 dilution on the plates of Robena iron bacteria. This occurred when filtrates from all three sites were tested against their respective bacteria. The plates of F. sulfo, while not as clear of bacteria as those of Robena and Karen, still showed some clear areas and plaques. Filtrates appeared to have a greater or lesser inhibiting effect on one or another strain of bacteria, but differences were not great.

The filtrates were also tested against bacteria in liquid synthetic iron media. It was observed that although ferrous iron was oxidized, the supernate liquid remained clear for varying periods of time in some tubes. The bacterial control tubes not only oxidized ferrous iron in two days, but also became cloudy. It seems reasonable to conclude that tubes with a clear supernate showed some evidence of partial bacterial inhibition. All results are shown on Table 14.

Three Wildwood filtrates partially inhibited Karen iron bacteria for 10 days, while Robena iron bacteria were partially inhibited for 5 days. No evidence of clearing was seen in tubes of stock cultures of F. sulfo and filtrates. It appeared that the phage or some other agent was inactivating some of the iron bacteria. The remaining iron bacteria developed a resistance to the inactivating agent and started multiplying again to oxidize the iron.

TABLE 14 - DRAINAGE SOURCES TESTED AS FILTRATES AGAINST IRON BACTERIA (1)

Source of Filtrate	Samples Taken at Various Times	Test Iron Bacterium	Solid Iron Agar Medium Lowest Filtrate: Bacterium Ratio Showing Plaques	Liquid Iron Medium (2) Inhibition of Iron Oxidation Days
Wildwood Waters	1	Karen Mine - F. sulfo	1:1000	10
Wildwood Waters	1	Robena Mine - F. sulfo	1:100	5
Wildwood Waters	2	Robena Mine - F. sulfo	1:1000	0
Wildwood Waters	1	Stock-Ferrobacillus sulfooxidans (3)	1:100	0
Wildwood Waters	2	Stock-Ferrobacillus ferrooxidans (3)	No plaques at 1:1 dilution	0
Wildwood Waters	2	Stock-Ferrobacillus ferrooxidans (4)	No plaques at 1:1 dilution	0
McCormick Drainage	1	Stock-Ferrobacillus ferrooxidans (4)	1:100	2
McCormick Drainage	1	Stock-Ferrobacillus sulfooxidans (3)	No plaques at 1:1 dilution	0
McCormick Drainage	1	Karen Mine - F. Sulfo	No plaques at 1:1 dilution	0
McCormick Drainage	1	Robena Mine - F. Sulfo	No plaques at 1:1 dilution	0

(1) Iron bacteria isolated from Karen and Robena mine sites were tested.

(2) Synthetic iron medium I as described on Table 1 was used for the iron oxidation tests. This medium was used as it more closely resembles true acid drainage than A or B media.

(3) - (4) These iron bacteria cultures were obtained from the American Type Culture Collection and are referred to as stock.

The Wildwood filtrates previously tested against Karen and Robena bacteria and showing partial inhibition of iron oxidation were retested in liquid media; as before, the ferrous iron was partially oxidized while the supernate remained clear. Aliquots were plated on iron agar. Aliquots from Karen and Robena control tubes were also plated at the same time. In both cases, a viable colony count showed that the filtrates reduced the bacterial counts in half.

Viable counts of 8 x 10^3 and 6 x 10^3 cells per ml were obtained from the Robena and Karen iron bacterial controls respectively, while the count from the Robena iron bacteria with filtrate was 5 x 10^3 cells per ml; the count from the Karen iron bacteria and filtrate tube was 3 x 10^3 cells per ml.

Higher cell counts should have been obtained but the iron agar plates were not freshly prepared and aliquots were plated 6 days after inoculation of tubes. This is beyond the peak growth for iron bacterial controls [2-3 days].

Fresh drainage sources were collected from Wildwood and McCormick areas and two-liter quantities of filtrates prepared against *F. sulfo*, *F. ferro* and Robena bacteria. This was done in an effort to produce better inhibitors against these bacteria. A three-liter quantity of effective inhibitor was previously obtained against Karen iron bacteria. See Table 14, sample 1.

Filtrates were prepared and tested against stock strains of *F. sulfo* and *F. ferro*. A filtrate was also tested against Robena bacteria. Although none of the fresh filtrates were effective in liquid media, a high degree of effectiveness was demonstrated against Robena bacteria by a Wildwood filtrate on solid agar plates. The filtrate inhibited most of the Robena bacteria even when diluted as high as 1000 times, then tested against Robena bacteria at a 1:5 filtrate to bacteria ratio. This data is also shown on Table 14. However, none of the recent sources showed inhibition against stock strains of *F. sulfo* and *F. ferro* on solid media.

A two-liter quantity of filtrate active against Robena bacteria was stored in the deep freeze and was later tested against Robena bacteria in flooded systems and troughs.

All Wildwood and McCormick waters were also tested directly against iron bacteria. Small numbers of plaques were sometimes obtained, but no inhibition of iron oxidation in liquid media was seen.

Various areas along the banks of a stream were tested frequently at the Wildwood mine throughout the project. Coal refuse lined the banks of the stream and the stream bed was composed of fine coal silt. It was found that a naturally occurring inhibitor appeared frequently during the late spring and summer months of 1968 and 1969. During the winter months, the inhibitor was not found. The pH was almost invariably somewhere near neutrality. Alexander, pp 26-27 (1967), has stated that most microorganisms are mesophiles (bacteria that grow best at 25°-35°C). The mesophilic types constitute the bulk of the soil bacteria, but certain mesophilic species develop best at temperatures below 20°C. These are cold-tolerant species. Usually, the viable population is greatest during the spring and autumn. A population burst occurs in the spring as the soil becomes warm and organic materials become accessible. The population commonly diminishes in winter and remains in a state of biological inactivity. The seasonal changes in numbers of bacteria are closely related to fluctuations in moisture and temperature. Alterations in temperature and moisture during the year may influence the bacteria directly. Climate factors may in part operate indirectly through surface vegetation which is the source of carbonaceous-nutrients reaching the microflora.

The McCormick area drainage was not tested as often as Wildwood because it is a very small mine making it difficult to obtain large volumes of water for inoculation.

Very recent testing of Wildwood water was conducted using serial dilutions of both drainage and filtrate. The tests were conducted in parallel and plated on iron agar. Tests results showed that both drainage and filtrate were effective against Robena iron bacteria (F. sulfo) at a ratio of filtrate to iron bacterial culture of 1:100. Clear areas of lysis were seen on the plates at 1:5 and 1:10 dilutions. Large clear areas of iron bacterial lysis were not seen at 1:100 dilutions, but numerous small clear plaques were present. At this time, July 1969, Wildwood waters were added to Robena mine test site. Tank trucks carrying 13,500 gallons were loaded with water from Pine Creek at the Wildwood mine. This is in excess of the amounts spelled out in our proposals: "115 gallons for a flow of 20,000 gpd" in the first draft and "as much as 10,000 gallons for a flow of<100 gal/min." The flow from the gob region in Robena was not known with a high degree of accuracy, but probably is less than 100 gpm or 144,000 gpd. Dosage was thus one hundred thirty five times the flow per minute compared with laboratory dosage as low as 100 times the flow per minute. However, it was not believed that Wildwood inhibitor was concentrated, as a 1:100 dilution against Robena iron bacteria is still fairly dilute and will probably not be effective under actual mine conditions.

Because of erratic results as to when the inhibitor appears and because it was not effective at high dilutions, it was believed that further testing of Wildwood inhibitors was necessary. It was hoped that the inhibitor could be identified, purified, then concentrated so that only small quantities would be needed to seed the Robena and Karen mine test sites.

Concentration of Phage-Like Inhibitors by Eluting Lysed Plates of Iron Bacteria

Three-liter quantities of Wildwood filtrates specifically inhibitory to Karen and Robena bacteria were obtained. These filtrates were tested against their specific bacteria on plates and in liquid iron media and found to be effective in low dilution as before. These two filtrates were designated as the original filtrates and were kept stored in a deep freeze until aliquots were removed for further testing and electron microscope studies.

An attempt was made to develop more concentrated material by plating at low dilution with iron bacteria. Thirty-six plates were used for each filtrate. After incubation the plates were eluted using synthetic liquid iron media. This was followed by low speed centrifugation tnen millipore filtration. Again the filtrates (about 200 ml each) were frozen until used. These transfers (T-1) filtrates were tested against their specific iron bacteria on overlay plates and in liquid iron media.

Both the original and T-1 filtrates were tested against bacteria on plates. In these tests the filtrates were progressively diluted from 1:5 up to 1:10^6 then tested against the same quantity of bacteria, i.e., 0.4 ml iron bacteria and 0.1 ml filtrate. It was found that the filtrate active against Karen iron bacteria was effective up to a ratio of 1:100 filtrate to bacterial culture. At this ratio only a few bacteria grew out on the plates. The filtrate active against Robena iron bacteria was less effective as only a 1:10 ratio inhibited bacteria. The T-1 transferred material showed about the same results. Liquid tube tests done in triplicate showed incomplete iron oxidation for six days beyond the controls when using the original filtrate against Karen and Robena iron bacteria. The T-1 filtrates showed incomplete oxidation for only a few days. With incomplete or partial iron oxidation the liquid portion of the tube remained mostly clear and the sediment was white (unoxidized) with a small amount of orange (oxidized) iron. The iron bacterial controls showed complete iron oxidation as the liquid became entirely orange and sediment was all orange.

It was not known why the filtrates inhibited iron bacteria for only six days in liquid cultures. It may be the ionic environments was not optimum for the adsorption of phage to their host cells. Adams, p-143 (1959) has stated that the rate of phage adsorption is depressed markedly by higher or lower concentrations of salts. The iron bacteria could also have developed resistance to the phage, or other inhibitor, which may not be a phage.

Concentration by Ultracentrifugation

Filtrates were subjected to 2 cycles of differential centrifugation, alternating speeds of 40,000 xg and 2000 xg for 8 hours. This was done in order to determine if potency of inhibitors could be increased. This procedure can be used to purify and concentrate phages. Similar procedures have been used by Adams, (1959), Bradley, p-234 (1965) and Zimmerer, (1969). The concentrates were then tested against the iron bacteria using aliquots of various dilutions on iron agar overlay plates.

In general, increases in potency were noted with some exceptions. It was found that a filtrate originally active against Robena bacteria was increased in potency from a 1:10 dilution to a $1:10^3$ dilution. The filtrate originally effective against Karen bacteria at a 1:100 dilution was increased to a $1:10^3$ dilution using the concentrate. Some filtrates originally show no effect on the stock culture of _F. sulfo_, the concentrated material showed plaques even when diluted to $1:10^4$. However, no clear areas of mass bacterial lysis was seen on agar plates. An _F. ferro_ concentrate gave the same results as the _F. sulfo_ concentrate. In liquid media however, the concentrated material did not inhibit any of the strains of bacteria.

Electron Microscope Studies

Aliquots of the Wildwood filtrates were ultracentrifuged at 37,000 xg for one hour and the residues examined in the electron microscope. This method has been used by Zimmerer (1969) to concentrate phages. Photographs were obtained but no biological agent was detected. Plates containing inhibited iron bacteria were also examined and a stalked species of bacteria was photographed from the Karen filtrate inhibitor. This suggested that some genera of bacteria such as Caulobacter may be inhibitory to iron bacteria.

Further Testing of Filtrates to Identify Phage-Like Inhibitors

It was strongly believed that every effort must be made to discover the basis of the inhibitory action of filtrates, particularly from the Wildwood sources. The fact that inhibitory material is heat labile and does not lose activity by dilution supports the view that it is of biological origin. The filtrates showing inhibitory action against iron bacteria as cleared (lysed) areas on iron agar plates were obtained from neutral mine waters and drainages. The Wildwood and McCormick mines have been in operation for 20-30 years and any lime in the rock formation should have been leached out. Most mine areas become acid after about five years as lime is leached out. Something has kept the drainages from these mines neutral and free of iron bacteria, although occasional samples contain iron bacteria. The iron bacteria-inhibiting material from these areas may thus be a biological agent. It was felt necessary to concentrate large quantities of filtrate from a Wildwood mine area source. Subsequently, an 8 liter quantity of filtrate previously found to be effective against Robena iron bacteria was prepared from a Wildwood source. This liquid was concentrated by freeze-drying, then examined using the electron microscope. It was hoped that a biological organism could be found. A large quantity of concentrate was obtained from four-liters of material.

The powder was re-suspended in water, centrifuged at low speed and the precipitate removed. The supernatant was ultra-centrifuged at 37,000 xg for four hours and electron microscopy done on the sedimented material. Nothing was seen except amorphous debris.

Aliquots of the freeze-dried powder were inoculated into various liquid media in order to determine if a microorganism could be isolated. No growth was present in media designed for the isolation of fungi, Streptomyces, iron bacteria and sulfur bacteria. The dissolved powder did not inhibit iron bacteria in liquid or on agar plates. It was evident that the freeze-dried powder had no biological activity and it may be that freeze-drying may have been responsible.

Testing for an Autocatalytic Inhibitor

It had been suggested that some of our previous positive inhibition results with Robena and Karen coal piles might be due to an autocatalytic inhibitor. This inhibitor is developed in the death phase of microorganisms; and, when applied to viable organisms, it caused them to produce more of the inhibitor and induces death.

Death was induced in all strains of iron bacteria by homogenizing cultures in a Waring blender with glass beads. The disrupted cultures were millipore filtered and the filtrates tested against fresh cultures of iron bacteria. Testing was performed in liquid synthetic iron solution and on solid iron agar plates. The results were negative in that the filtrates did not inhibit fresh iron bacteria in liquid cultures, nor did plaques or cleared areas appear on agar plates. The filtrates were tested by themselves in liquid iron and on agar plates in order to determine if the homogenizing and filtering removed and killed the iron bacteria. None of the filtrates oxidized iron in liquid cultures and no bacterial iron colonies appeared on agar plates. The conclusion appeared to be that autocatalytic inhibitors were not formed in cultures of iron bacteria.

In order to rule out the possibility that filtrates producing plaques effective against iron bacteria were caused by an organic product or inorganic effects, a Wildwood filtrate previously found to be inhibitory was boiled, then retested against iron bacteria. No inhibitory plaques were seen indicating some kind of biological activity was originally present in the filtrates.

Conclusions

The results tend to disprove the presence of phage as an iron bacteria inhibitor. Hundreds of samples were collected from numerous acid and neutral mine areas and tested for the presence of phage. Iron bacteria inhibitors were never found in samples obtained from acid mine areas. Inhibitory effects could sometimes be obtained from drainages located in neutral or alkaline mine areas, but inhibition appeared to be caused by chemical agents as filtrates could not be transferred without losing their inhibiting action. In a few of the neutral mine areas, drainages prepared as filtrates appeared to be biological in nature as they could be transferred by filtration and retained inhibitory activity against iron bacteria. In most cases, inhibition could not be proved to be of a biological nature. Although hundreds of electron micrographs were obtained of filtrates, lysed areas on agar plates, and various types of concentrated material, no photographs of phage were ever obtained. The inhibitory material could be transferred up to a certain point, was heat labile and could be filtered. By these methods it could be concentrated but not to a great degree. As a result of lack of success in great efforts to concentrate the material, (ultracentrifugation and elution of many plates producing only small quantities of inhibitory material), it is doubtful that large quantities could be produced by these methods. In addition, the material can only be obtained at certain times of the year. It is therefore, unlikely that an unconcentrated natural inhibitor could be economically applied to mines. However, some electron photographs of iron bacteria lysed plates were obtained in which Caulobacter could be seen. As this microorganism can be filtered, is harmless and may be the cause of inhibition of iron bacteria, studies were conducted to find if this microorganism was inhibitory to iron bacteria on the premise that if Caulobacters act as inhibitors against iron bacteria, they could be isolated, then easily mass-produced and inoculated into mines.

SECTION 12

CAULOBACTERS

BACKGROUND

Previously, electron microscopy showed that Caulobacters or closely related microorganisms were present on iron agar plates in which lysis of iron bacteria was seen as plaques. The plates had been seeded with iron bacteria and filtrates prepared from Wildwood and McCormick waters. No Caulobacter or any other microorganisms except iron bacteria were seen on the control plates containing iron bacteria but no Wildwood or McCormick waters. It was reasoned that Caulobacter or some closely related microorganism might be responsible for inhibitory effects on iron bacteria.

Caulobacters have stalks which are extermely small in diameter and can pass through very fine millipore filters. The diameter of the stalk as given by Poindexter, p-234 (1964) is 0.2 micron. Zimmerer, (1969) has reported that Caulobacters can pass through 0.2 micron millipore filters as he has found them in electron micrographs. The chances of finding Caulobacters in otherwise sterile filtrates is particularly good if suction (negative pressure) is used to prepare filtrates as this tends to pull the Caulobacters, stalk first, through the membrane filter. The microorganisms are harmless and have often been isolated from lakes, streams, soil, water lines and tap water faucets. The nature of the holdfast stalk is still unknown, but may attach to solid objects. They also have a life cycle in which free swimming forms and stalked forms predominate at one time or another. If stalked forms can attach to solid objects, there does not appear to be any reason why they could not attach to coal; and, since they can live in waters containing only minute amounts of peptone, there does not seem to be any reason why they could not live in coal environments. Generally, Caulobacters live at neutral pH, but, they could probably be adapted to a low pH or live in acid waters as part of their life cycle. This has been reported by Gerencser, (1969). It was also thought they may attach to iron bacteria as there have been reports in the literature of this happening to other bacteria, Poindexter, (1964). Caulobacters may also compete for the food supply and reduce the number of iron bacteria.

Details of experimental results regarding the static testing, adaption, testing under dynamic conditions in the various microbiological systems will be discussed in detail in the following section.

Initial Attempts at Testing and Adaptation of Stock Caulobacter

A stock culture of a single strain of Caulobacter, which normally grows in pond water, was obtained from the American Type Culture Collection and grown in a nutrient solution recommended by Poindexter, (1964). The liquid standard medium was designed to allow good growth of Caulobacter but other microorganisms could also grow. The liquid was defined as standard medium and is composed of tap water with 0.2% peptone, 0.1% yeast extract, and 0.02% magnesium sulfate. For solid standard agar, 1.0% agar was added and allowed to harden in petri dishes. As originally isolated, this microorganism was obtained from natural pond waters with only 0.01%peptone added.

After several transfers in liquid standard medium, this type III Caulobacter species was transferred directly to the three modifications of iron media, synthetic I, A and B.

Stained slides were prepared from liquid aliquots of the three iron media to which the Caulobacter had been transferred. The slides showed what appeared to be detached stalks. A few non-stalked cells were also present. However, this did not indicate that Caulobacter was actively growing in the iron media as these microorganisms could simply have survived as they carried over nutrient from their original growth medium. The Caulobacter was tested against all strains of iron bacteria in the liquid iron media.

Results showed that Karen iron bacteria were prevented from oxidizing ferrous iron four days beyond the control tubes of Karen iron bacteria only. But this delay in iron oxidation occurred only in iron medium A. A stock culture of F. sulfooxidans was prevented from oxidizing iron for 9 days in liquid iron medium A. However, the Caulobacter did not prevent the Robena iron bacteria from oxidizing iron in any of the iron media in which it was tested.

Electron micrographs were taken from aliquots of the tube containing unoxidized iron medium A inoculated with Caulobacter and the stock culture of iron bacteria, F. sulfooxidans. Electron micrographs were also taken of the iron bacterial control tube containing F. sulfooxidans which had oxidized iron in medium A.

The electron micrographs were taken from aliquots of the tube containing unoxidized iron medium containing iron bacteria and Caulobacter. Caulobacter was not seen in aliquots from the control tube medium containing iron oxidized by the iron bacteria.

The results showed that Caulobacter was not present as a contaminant in the liquid iron nutrient solution, or as a mixed contaminant being carried along upon transfer of supposedly pure cultures of iron bacteria. However, the results of the electron micrographs did not give enough evidence to prove Caulobacter inhibited iron bacteria in liquid cultures. The Caulobacters were grown initially in a neutral solution which was organic in nature, containing peptone and yeast. Upon direct transfer to the essentially inorganic iron medium, the Caulobacter cells could have carried over some of the organic material which may have partially inhibited the iron bacteria.

The stock Caulobacter strain was transferred a second time in like iron I, A and B then re-tested in triplicate against all the iron bacteria, again in the same iron media. Caulobacter was added to the iron media in a 1:1 and 5:1 ratio with each specific iron bacterium; i.e., stock cultures of F. sulfo and F. ferro, Robena (F. sulfo) and Karen (F. sulfo). The dilutions of Caulobacter and iron bacteria used were 1:100 in each synthetic iron medium of acid pH (3.6).

Results showed that Caulobacter prevented Karen iron bacteria from completely oxidizing ferrous iron for four days beyond the control tube containing Karen iron bacteria only. After the fourth day, iron became completely oxidized. Stock cultures of F. sulfo and F. ferro were prevented from oxidizing iron for three days. Increasing the ratio of Caulobacter over iron bacteria did not substantially prolong delay of iron oxidation by the iron microorganisms.

Aliquots from the partially oxidized tube of Caulobacter and Karen iron bacteria and fully oxidized Karen bacterial control tubes were transferred to iron agar plates. After growth occurred, Caulobacter could be seen as very small pinpoint colonies in the heavier growth of yellow iron bacterial colonies. The white pinpoint colonies appeared to have some inhibitory action as clear unoxidized halos (plaques) appeared around these colonies and also around some of the iron bacteria. The zones of inhibition on the iron agar plate containing Karen iron bacteria and unadapted stock Caulobacter is shown in Figure 17. The control iron agar plate of Karen iron bacteria without Caulobacter did not show any plaques.

Visual observation of synthetic iron agar plates containing Caulobacter showed sparse growth of these microorganisms which appeared as pinpoint white colonies. Aliquots obtained from liquid and iron agar plates, and prepared as stained slides, were examined under the microscope. Caulobacter could not be seen as typically stalked microorganisms. However, some atypical forms or contaminating microorganisms were seen. The characteristic appearance of Caulobacter

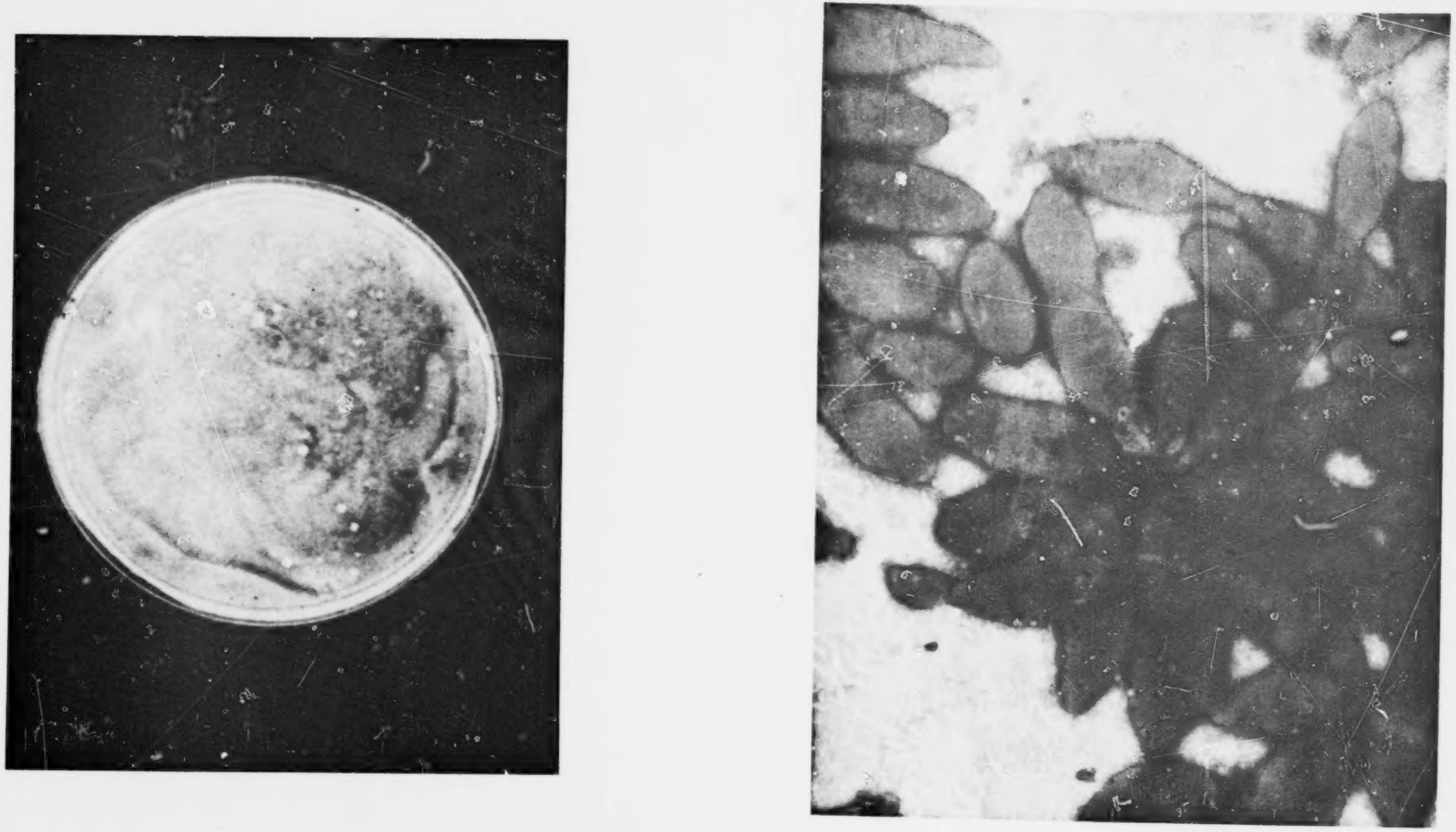


FIGURE 17 ZONES OF INHIBITION ON AGAR LAYERS (LEFT) AND ELECTRON MICROGRAPH OF CAULOBACTERS (RIGHT)

as seen in an electron microscope (taken from the original stock culture grown in standard medium)is shown in Figure 17. In iron medium, detached stalks could be seen. It was not known at that time if the bacterial form of Caulobacter changed upon transfer from its original standard medium to iron medium, if iron in the medium obscured their typical appearance, or if Caulobacter just did not grow in iron media.

Only one stock culture of Caulobacter had been studied up to that time. It was hoped that Caulobacters could be isolated from previously inhibitory waters from Wildwood and McCormick mines and that they would be inhibitory to iron bacteria. This will be discussed in the following section.

Isolation of Caulobacter Strains from McCormick and Wildwood Waters

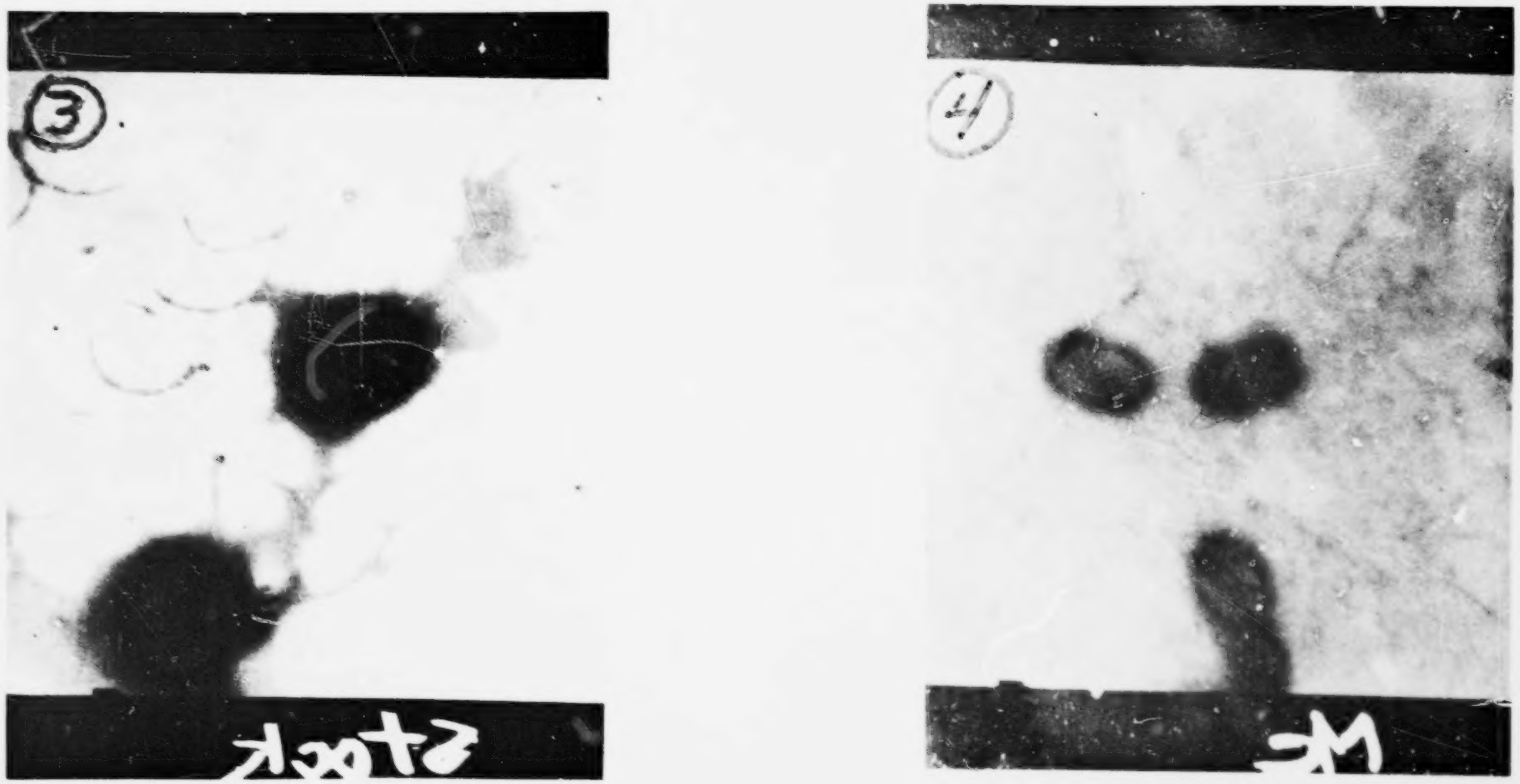
In an attempt to determine the nature of inhibitory waters, six strains of Caulobacter were isolated from neutral drainage sources of Wildwood, and three strains from the McCormick mine areas. The Caulobacters were grown by adding small quantities of peptone, (0.01 percent) to the neutral waters, incubating and plating on standard agar. Colonies grown on agar were picked and stained to identify; then each Caulobacter strain was picked and transferred to fresh agar plates to obtain pure strains. The strains were different as indicated by their morphology on agar plates and microscope appearance.

All strains of Caulobacter were picked from agar plates and placed directly into tubes of iron medium A (pH 3.6), incubated and tested against the iron bacteria. The results were negative in that none of the Caulobacter strains prevent bacterial iron oxidation.

Stained slides were prepared of all the isolated Caulobacters inoculated into acidic iron media after being transferred from their naturally neutral waters. The typical stalked form of Caulobacter could not be seen in the microscope. It was thought that Caulobacter might have a better chance to adapt to acid iron conditions if they were to be progressively adapted to iron media containing more iron and acid since results showed they could not immediately adapt to synthetic iron media having a much lower pH than their original environment. This will be discussed in the following section.

Initial Attempt to Adapt Caulobacter Strains to Iron Media

Mixed Caulobacter strains isolated from McCormick, mixed Caulobacter strains isolated from Wildwood, and the stock Caulobacter obtained from the American Type Culture Collection (A.T.C.C.)



A - Stock ATCC

B - From McCormick Effluent

FIGURE 18 STOCK AND MCCORMICK ADAPTED CAULOBACTER CULTURES -
STANDARD AGAR PLATES

were added separately to iron medium A. This differed from the original medium (Table 1) in that iron was not added to the salt medium and the pH was 7.0. The mixed Caulobacter strains were added to tubes at a dilution of 1:100 in 10 ml of the salt medium. Peptone was added in the amount of 0.01 percent to each 10 ml of medium. Tubes were incubated at 27° C for one week, aliquots placed on slides which were then stained using a flagella stain to observe stalked microorganisms. All Caulobacter strains were seen as typical stalked cells.

Transfers were made from the salt medium at pH 7.0 to a modified iron medium, again at 1:100 dilution in 10 ml of modified iron medium. The modified medium contained its normal amount of salts as shown in Table 1, except that 100 ppm of Fe II was used instead of the usual 200 ppm. Sulfuric acid was not used and the pH was 5.0. Peptone was added in the amount of 0.01 percent. The tubes were incubated for one week, then flagella stains prepared as before. Microscopic examination of the stained slides showed mostly bacterial cells appearing as rods and detached stalks. Only occasional typical stalked Caulobacter cells were seen.

Aliquots of Caulobacters growing in the modified iron media were again transferred to the same media, incubated for a week and stains prepared as before. The appearance of the Caulobacters was the same as noted previously, i.e., detached stalks and bacterial cells.

Aliquots from each of the three sets of Caulobacters (Wildwood, McCormick and stock) were removed and tested against iron bacteria in the original iron medium, medium A as described in Table 1. A 1:1 Caulobacter to iron bacteria ratio was used. Iron bacteria and Caulobacter were diluted 1:100 in 10 ml of the original iron II medium A (Table 1).

Results showed that iron was oxidized in 14 and 8 days in iron medium A (Table 15) inoculated with Karen and Robena iron bacteria respectively, and McCormick obtained Caulobacter strains. Iron II was oxidized in 2 days in all iron bacterial control tubes containing iron bacteria and media. The negative controls containing synthetic iron media only showed that iron II remained unoxidized throughout the test period of 14 days. Results are shown in Table 15.

Aliquots were removed from all modified iron media (pH 5.0) containing the Caulobacter strains, and transferred in the same quantities as before to synthetic iron medium A at pH 3.6, the composition of which is shown in Table 1, the only difference being that 0.01 percent peptone was added. Tubes were incubated, aliquots removed and stained as before.

TABLE 15 - TESTING ADAPTED CAULOBACTERS AGAINST IRON BACTERIA

Source of Caulobacters	Medium Modifications (1)	Test Iron Bacteria (2)	Oxidation of Fe II (3) Days
McCormick Strains	Synthetic A salts; 100 ppm Fe II, pH 5	Karen	14
		Robena	8
Wildwood Strains	Synthetic A salts; 100 ppm Fe II, pH 5	Karen	7
		Robena	7
A.T.C.C. - Type III	Synthetic A salts; 100 ppm Fe II, pH 5	Karen	6
		Robena	3
McCormick Strains	Synthetic A, 200 ppm Fe II, pH 3.6	Karen	11
		Robena	9
Wildwood Strains	Synthetic A, 200 ppm Fe II, pH 3.6	Karen	4
		Robena	4
A.T.C.C. - Type III	Synthetic A, 200 ppm Fe II, pH 3.6	Karen	4
		Robena	3
McCormick Strains	Synthetic A, pH 3.6 with coal and pyrite	Karen	9
		Robena	8
Wildwood Strains	Synthetic A, pH 3.6 with coal and pyrite	Karen	2
		Robena	2
A.T.C.C. - Type III	Syntheitc A, pH 3.6 with coal and pyrite	Karen	4
		Robena	3

(1) Details of the iron media compositions have been described in the written commentary.

(2) Caulobacters were grown in their adaption media and tested against iron bacteria in synthetic A iron media of pH 3.6 as described in Table 1.

(3) The Karen and Robena iron bacterial controls were inoculated with each set of experiments; Fe II was oxidized in 2 days. The negative controls remained clear, and unoxidized throughout each test period.

Microscopic examination of the slides showed very few detached stalks and bacterial cells. The Caulobacters were tested against Karen and Robena iron bacteria in medium A, pH 3.6 in the same manner as previously described. The results shown in Table 15 indicate that McCormick strains prevented Karen iron bacteria from oxidizing Fe II for 11 days and Robena bacteria for 9 days, beyond the two day Fe II oxidation times of Karen and Robena bacterial controls. The negative bacterial controls remained unoxidized throughout the test.

The Caulobacters incubated in the original iron medium A (Table 1) were transferred to the same medium except that iron was omitted. The medium was acidified to pH 3.5 with sulfuric acid. Two gm of coal and 1 gm of pyrite were added to each tube containing 10 ml of medium. Coal and pyrite were added to supply iron and sulfur. After incubation for two weeks, aliquots were removed and stained as before. Caulobacters were not seen as cells or stalks. However, coal, pyrite and iron were present, as debris, to such an extent that very little else could be seen.

Aliquots were tested against iron bacteria in acid iron medium A (Table 1) as previously stated. The McCormick strains prevented Karen and Robena iron oxidation for about 9 days, Wildwood strains did not prevent iron oxidation while the A.T.C.C. strain of Caulobacter prevented Fe II oxidation for only a day or two beyond the iron bacterial control tubes.

Transfers were made again from acidified medium A with coal and pyrite added to the same medium at a 1:100 dilution. After incubation, aliquots were tested against the iron bacteria. None of the Caulobacter strains prevented bacterial iron oxidation for more than a few days beyond the iron bacterial controls.

A line sketch of the Caulobacter adaptive procedures is given in Table 16.

In summary, six combined strains of Caulobacters isolated from Wildwood waters, and one strain of stock (A.T.C.C.) Caulobacter did not appear to adapt well to acid/iron conditions. Three combined strains of Caulobacters obtained from the McCormick mine waters showed partial adaption, but small quantities of peptone appeared to be necessary for adaption to acid/iron environment.

Inability to adapt may have been caused by transferring Caulobacters in test tube quantities. It was felt that the strictly aerobic Caulobacters did not get enough oxygen to allow them to acclimatize to unnatural growth media. In addition, poor inhibiting powers may have been caused by testing small populations

TABLE 16 - STEPS IN ATTEMPTS TO ADAPT CAULOBACTERS

pH 7.3 Mine Waters
containing 0.01 percent
peptone
1:100 dilution

↓

Iron medium salts A-
pH 7.0 No Fe II -
0.01 percent peptone
added
1:100 dilution

↓

Iron medium A - pH 5.0
100 ppm Fe II added
0.01 percent peptone
added
1:100 dilution

↓

Iron medium A - pH 3.6
H_2SO_4 added - 200 ppm
Fe II added - 0.01 percent peptone added
1:100 dilution

↓

Iron medium A - pH 3.6
H_2SO_4 added - 200 ppm Fe
II added - 0.01% peptone
added
1:100 dilution

↓

Iron medium A - pH 3.5
No Fe II added - H_2SO_4
added - Iron and pyrite
added. No peptone added
1:100 dilution

↓

Iron medium A - pH 3.5
No Fe II added - H_2SO_4
added. Iron and pyrite
added. No peptone added.

of Caulobacters against concentrated cultures of iron bacteria. The viable cell counts of iron bacteria tested were on the order of 1 x 10^9 cells per ml. This has been shown in a previous report by Shearer, R.E. and Everson, W.A., p-10 (1965).

It was hoped that further attempts to adapt Caulobacter might be more successful if larger flasks and greater quantities of liquid nutrient medium were used to provide more surface area. Actively aerating and constant shaking might provide better environmental conditions. If full adaption could be attained (with peptone obtained from the acid bacteria or substituted with the organic content of coal), then further testing would be conducted in vertical flooded and horizontal systems. These systems have smaller iron bacterial populations which simulate natural conditions in a coal mine environment.

Since there were too many individual strains of Caulobacter to be adapted and tested, a decision had to be made as to how to proceed. The stock culture of Caulobacter was used as it had shown some initial inhibition against iron bacteria. In addition, mixed McCormick Caulobacter strains were used since it was not known which single strain was most effective. Mixed McCormick isolated strains were used instead of Wildwood isolated strains because only three strains of Caulobacter were obtained from McCormick water, while six strains were obtained from Wildwood water. Also many other types of microorganisms were isolated on standard agar plates from Wildwood waters. It was thought best to avoid other contaminating bacteria and fungi which might interfere with test results and give confusing data.

Further Attempts to Adapt Caulobacters to Acid/Iron Environments

The stock culture (A.T.C.C.) of Caulobacter and the mixed strains of Caulobacters isolated from McCormick neutral drainages were propagated in neutral sterilized tap water containing 0.01% peptone. These strains were transferred and gradually adapted to an acid/iron environment in 3-liter flasks containing only one liter of iron medium to allow a large surface area. Spargers were connected from a sterile air source and into the flasks beneath the liquid surface. The flasks were then shaken and aerated continuously. After several transfers through gradually increasing concentrations of iron and acid, the Caulobacters were cultured in a final medium of synthetic A (Table 1) acid mine drainage with the full amount of iron and a pH of 3.6. It was felt necessary to include small quantities of 0.01% peptone. Gradual adaption of the Caulobacters to acid/iron conditions were made in a similar manner as previously described for transferring Caulobacters in test tubes.

However, in present studies, larger quantities were used and cultures shaken and aerated.

These larger quantities of Caulobacters were tested against Karen and Robena iron bacteria and were found to prevent iron oxidation for about three weeks beyond the iron bacterial controls.

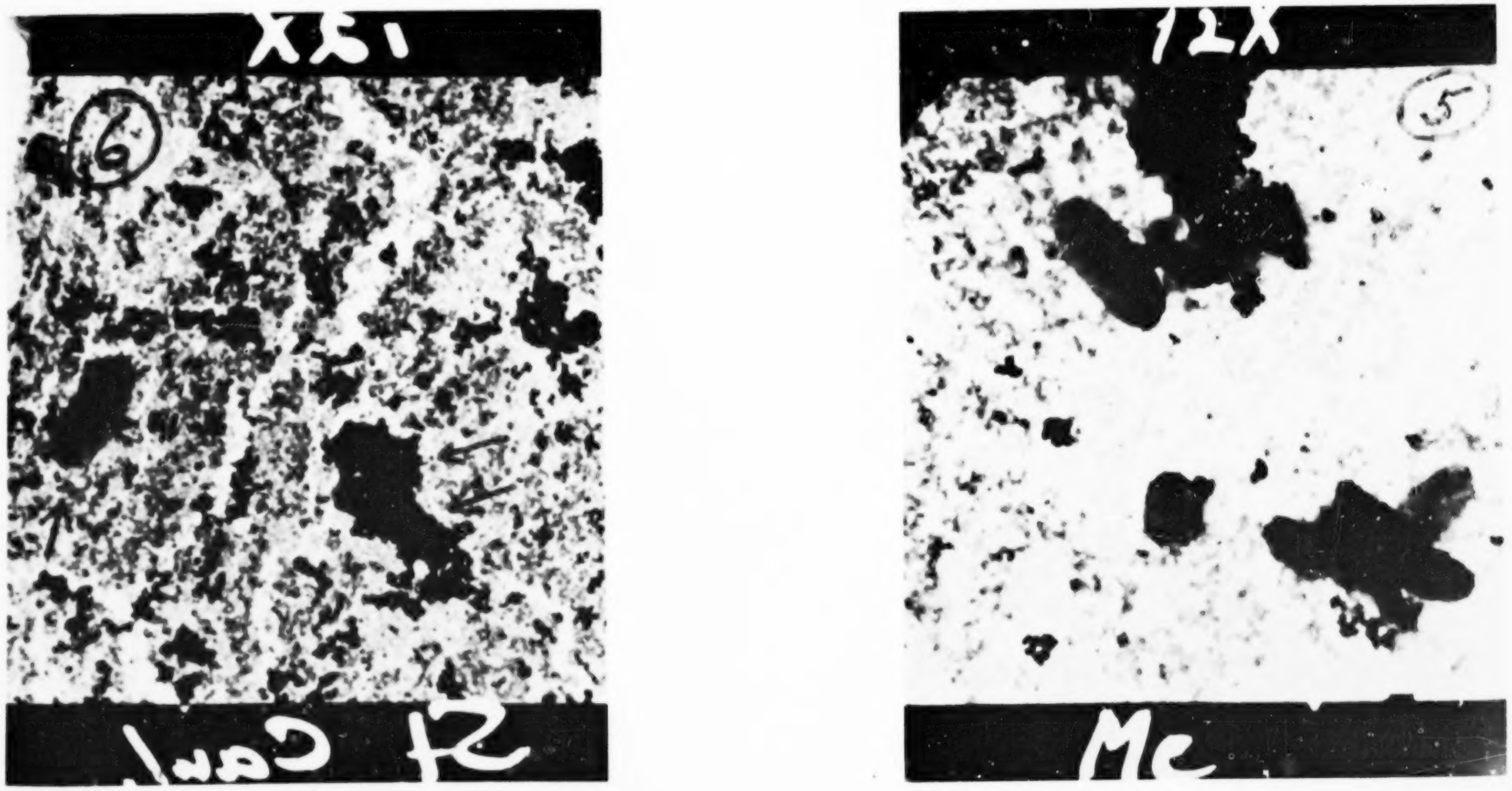
The stock (A.T.C.C.) and McCormick-isolated Caulobacters appeared to partially adapt to acid/iron environments; proof of this will be discussed under Electron Microscope Studies following.

Electron Microscope Studies

The liquid cultures of acid/iron-adapted McCormick and stock A.T.C.C. Caulobacters used to inoculate the the flooded vertical coal systems were transferred to standard agar plates. (The flooded systems will be described in detail later). These plates are the natural growth medium for Caulobacter. It was not known if acid/iron adapted Caulobacter bacterial growth would occur as this medium is organic and has a slightly alkaline pH. However, excellent growth was obtained from both Caulobacter cultures. These plate cultures were examined in the electron microscope. Figure 18 shows the microorganisms as they appear on standard agar plates from the stock A.T.C.C. and McCormick iron-adapted cultures.

The liquid cultures of stock and McCormick Caulobacter used to seed the coal piles were also examined. These cultures had been adapted to gradually increasing concentrations of iron and acid. In addition, a number of successive transfers had been made in the final synthetic acid drainage. Electron microscope studies showed that the stock and McCormick cultures were not contaminated. (See Figure 19).

Samples were later obtained from vertical flooded systems 8 and 9. Number 8 has had additions of what was thought to be iron-adapted Caulobacter from the stock A.T.C.C. culture, and from filtered McCormick mine neutral drainage. Container 9 served as a bacterial control tank and inhibitor was not added. Samples were withdrawn 50 days after the last addition of acid-adapted Caulobacter. Since these tanks were dynamic systems with constant water flow, any washout of inhibitor should have occurred unless it had become part of the normal flora of the coal system. The samples were analyzed using the electron microscope. Various kinds of bacteria were seen and photographs taken. Sample 9 (control) was characterized by having flagellated bacteria and crystalline materials. The predominant bacteria seen in effluents from Sample 8 (test container) were larger than the flagellated bacteria in the control and appeared in clusters



A - Stock ATCC

B - McCormick

FIGURE 19 ADAPTED CAULOBACTER CULTURES USED TO INOCULATE COAL

as shown in Figure 20. These bacteria appeared to be the right size for Caulobacter, 0.5-1.2 micron, but were without stalks or flagella normally seen with Caulobacter.

It can be seen that the same bacteria were present on the agar plates and from the liquid cultures. It also appeared that the same bacteria were present from effluents of the test container (#8) after fifty days. In order to determine if the original stock Caulobacter was truly Caulobacter, as obtained from A.T.C.C., electron micrographs were made of stock Caulobacter grown on standard agar from its original tap water-peptone liquid medium. It was found that the original stock Caulobacter used for adaptation to liquid synthetic iron medium was Caulobacter as seen in Figure 17.

Further work was done in attempts to prove that the stock and McCormick adapted seed cultures were pure (uncontaminated) and that the same bacteria were present in the cultures used to seed the flooded container number 8 as in the container itself.

Additional Microbiological Studies on Caulobacter

The stock and McCormick Caulobacter seed cultures were plated out on standard (Caulobacter) agar, Czapek's(Waksman, p-245 (1967) and Hesseltine, [1954]) (Streptomyces and fungi)agar and iron agar. Good bacterial growth appeared on standard agar, but scant growth was seen on Czapek's agar, and no growth recognized as iron bacteria appeared on iron agar.

Aliquots from flooded container 8 (test container) and 9 (bacterial control container) were plated out on the same media. The same type of colonies was isolated on standard agar from container 8 as were found from the stock and McCormick cultures used to seed the test container. An entirely different type of bacterial growth was obtained from container 9 on standard agar.

Aliquots from test container 8 (contains Robena iron bacteria and Caulobacters) and control container 9 with Robena iron bacteria only, did not show growth on Czapek's agar. Iron bacteria from both containers grew as colonies on iron agar plates. These results appeared to indicate that the same type of bacterium was present from both seed cultures which contained Caulobacter, and, also from container 8, but not 9. Electron microscope studies of colonies growing on standard agar plates and isolated separately from the adapted seed culture of McCormick, and stock Caulobacter as well as from container 8 had the same appearance. Short rods, 1 micron in length x 0.5 micron in width were seen. These bacteria were flagellated, appeared in clusters, but were without stalks.

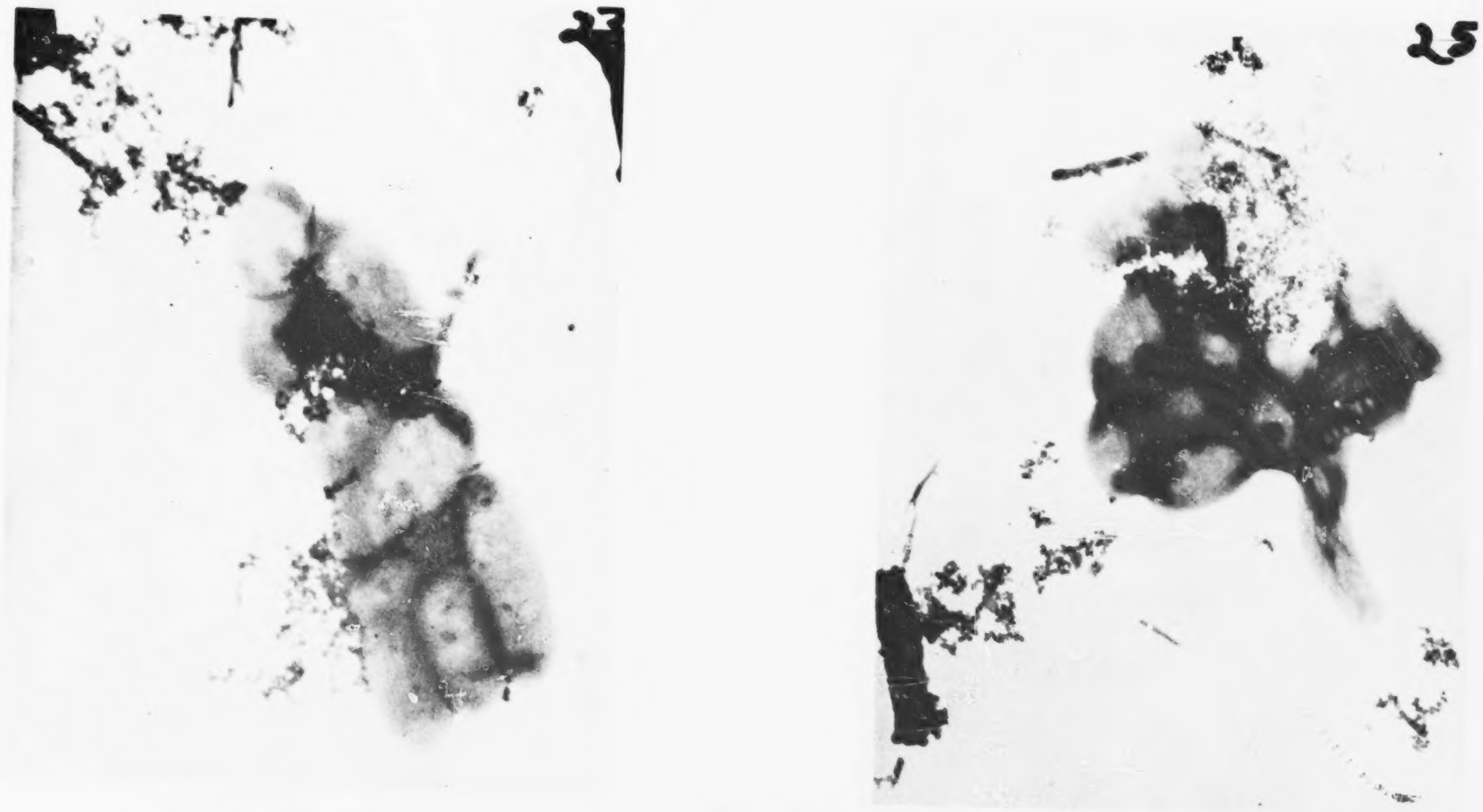


FIGURE 20 TWO ELECTRON MICROGRAPHS OF PREDOMINANT BACTERIA IN EFFLUENTS FROM CAULOBACTER - INHIBITED SYSTEMS

Summary of Electron Microscope Results

Indications were that the good results in decreased acidity at the flooded container number 8 were due to the addition of seed cultures from the stock or McCormick cultures. The microorganism responsible may be an adapted form of Caulobacter, although electron photographs do not show the typical appearance of Caulobacter. Of course, the Caulobacter may have died out under the extreme acid conditions and another microorganism may have become predominant.

Proving that the Caulobacters have adapted to such an extent as shown in the photographs is a long and tedious process and attempts were not made because of lack of time. It was believed that vertical flooded-system testing should be repeated with these cultures in order to clearly prove that they were responsible for the improvement in iron and acid conditions.

A summary of the major points concerning electron microscopy done on supposedly adapted Caulobacter cultures and their addition to flooded vertical coal systems is as follows:

1. Certain of the "Caulobacter" isolated from the McCormick mine and adapted Stock Caulobacter grown both at pH 3.5 and on Standard agar resemble some of the bacteria which were found to reduce iron oxidation and acid formation in coal.

2. Some of the Caulobacter of the original stock culture also resemble both the McCormick "Caulobacter" and the "adapted" Stock Caulobacter.

3. The similar appearance of these Caulobacters as seen with the electron microscope merely helps to confirm but does not unequivocally prove that these are Caulobacter species. Other toxonomic and characterizing data must be obtained.

Before we can definitely state that Caulobacter act as inhibitory bacteria, experiments need to be rigorously repeated, starting with the following:

1. Obtain pure A.T.C.C. cultures of Stock Caulobacter species.

2. Obtain pure cultures of McCormick Caulobacter from McCormick water.

These cultures should be obtained pure and kept in their natural growth medium (tap-water peptone) at slightly alkaline pH. Once pure cultures have been obtained, they should be again adapted to an acid pH with special care taken to avoid contamination. Samples should be adapted in triplicate so that comparisons of the resultant bacteria can be made. Once adapted to an acid pH, if possible, these cultures should be tested for their inhibitory properties in carefully controlled experiments with the vertical flooded coal systems. Experiments should also be performed in duplicate.

Inoculation of Adapted Caulobacters into Flooded Flowing Vertical Systems

Inoculations of Caulobacters into media containing iron bacteria gave some inhibition of growth of the iron bacteria as evidenced by delay of Fe II oxidation in earlier work. Cells of Caulobacters were also found previously in inhibited cultures by electron microscopy. It was of interest to determine if inhibition could be obtained in flowing systems having the Fe II or the sulphide content of coal and pyrite as the nutrient.

Vertical Flooded System

Two containers (8 and 9) similar to those used to study inhibition of Karen and Robena iron bacteria were set up to study effects of Caulobacters on the iron bacteria in vertical flooded systems. The acid-producing bacteria were established in these flowing systems containing high-sulfur coal refuse and pyrite as evidenced by iron bacterial viability counts and increased acidity of the effluents. Two hundred milliliters each of the Caulobacters, sp. Type III from the American Type Culture Collection and of Caulobacter cultures originally isolated from McCormick mine water, were added to one of the pairs of containers (8). These were the iron-adapted and batch-produced Caulobacters which had been grown in one liter amounts in synthetic mine drainage while shaking and aerating. The other container was free of Caulobacters as a control (#9). A second addition of Caulobacters was made to #8, thirty days after the first when the pH had risen to 2.9. A third addition was made 205 days after the first and results are shown on Table 17 and in Figures 21 through 32. Data on sulfates and iron were not taken beyond 154 days to preserve funds for more extended data on the significant acidity and pH.

Another test was started later using two containers (10 and 11). These results are also shown in Table 17 and in Figures 21 through 32 with some of the data limited to less than the approximate 250 days on acidity and pH due to paucity of funds at that time. Evidence of inhibition over at least some period of time is seen both in chemistry and microbiology of the systems. Statistical analyses of the data showing in-

TABLE 17 - VIABILITY OF ROBENA BACTERIA IN VERTICAL STREAMS WITH AND WITHOUT CAULOBACTER INHIBITOR

Days After Adding Caulobacter	Cells/ml #8 Caulobacter	 #9 No Caulobacter
0	30,000	1,300
2	7,900	10,000
9	10,700	8,000
17	30,000	30,000
23	2,000	3,000
30	100	2,000
37	100	5,000
45	2.900	3,500
51	3,600	55,000
58	8,000	43,000
65	700	10,000
72	35,000	150,000
80	2,200	23,000
88	800	8,000
94	3,000	5,000
102	1,400	10,000
109	1,000	2,000
116	6,000	8,000
123	1,700	9,200
129	1,800	3,000
137	300	1,000
144	700	-
151	300	<1,000
158	500	5,000
164	200	15,000
172	300	2,000
178	400	2,000
186	200	900
193	<100	1,300
200	<100	1,000

TABLE 17 - VIABILITY OF ROBENA BACTERIA IN VERTICAL STREAMS WITH AND WITHOUT CAULOBACTER INHIBITOR (continued)

Days After Adding Caulobacter	Cells/ml #11 Caulobacter	 #10 No Caulobacter
-6	1,180,000	1,950,000
2	670,000	1,000,000
9	4,000	5,000
16	120,000	80,000
23	300,000	58,000
29	300,000	30,000
37	328,000	43,000
44	10,000	10,000
51	17,000	8,000
58	<1,000	64,000
64	2,500	5,000
72	< 100	6,000
78	< 100	3,000
86	1,700	25,000
93	1,900	2,500
100	< 100	2,000

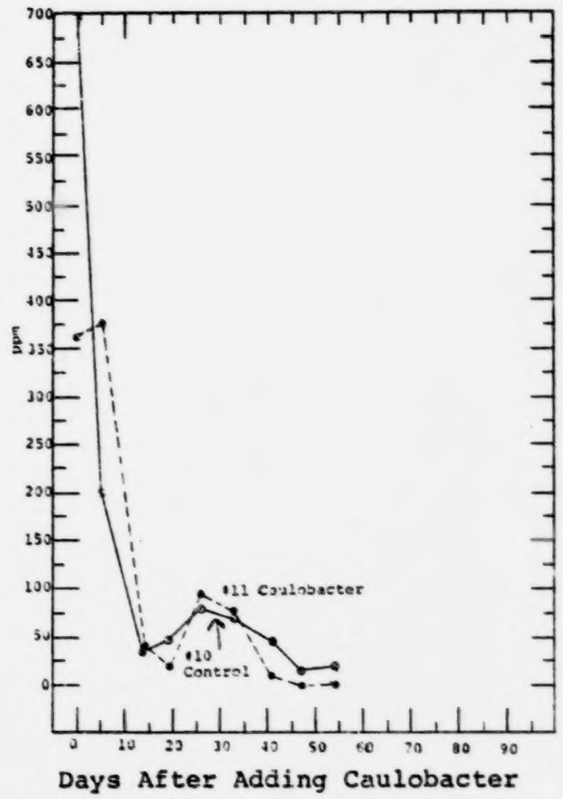


Figure 21 - Test 2

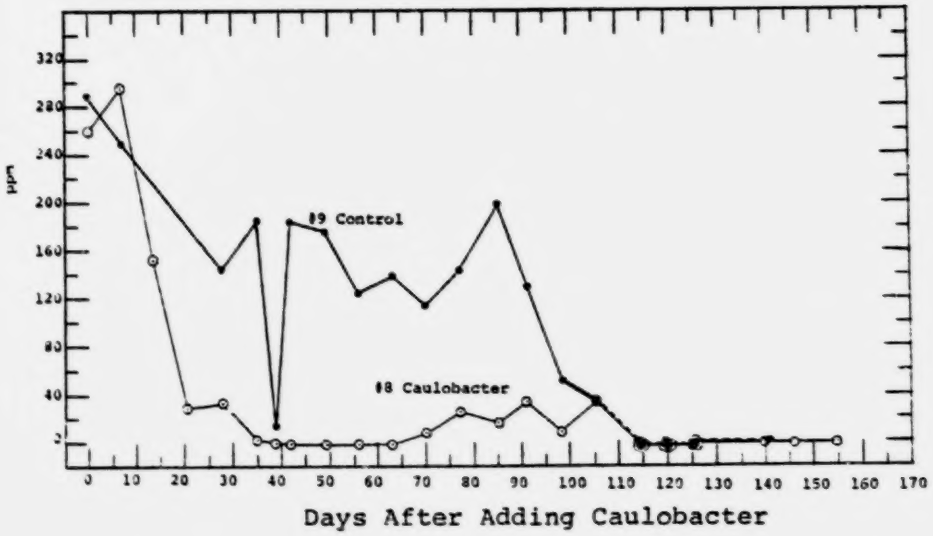


Figure 22 - Test 1

Effect of Caulobacter on Production of
Ferrous Iron by Robena Bacteria

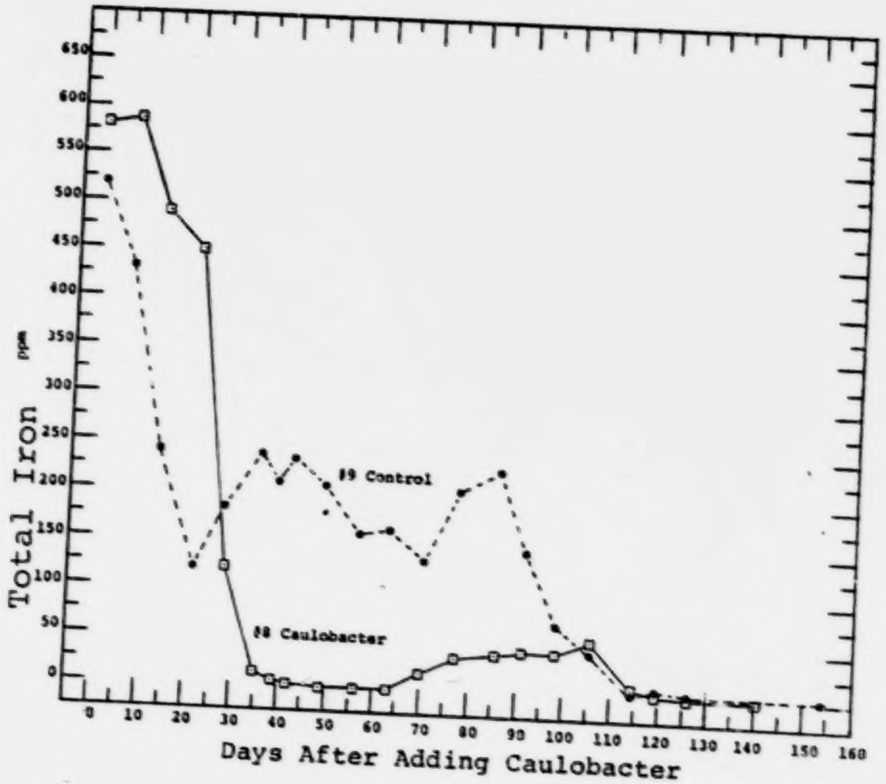


Figure 23 - Test 1

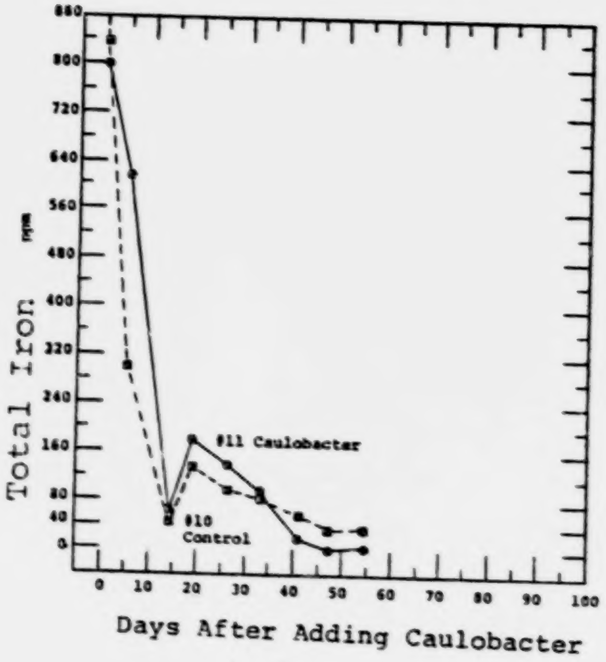


Figure 24 - Test 2

Effect of Caulobacter on Production of
Total Iron by Robena Bacteria

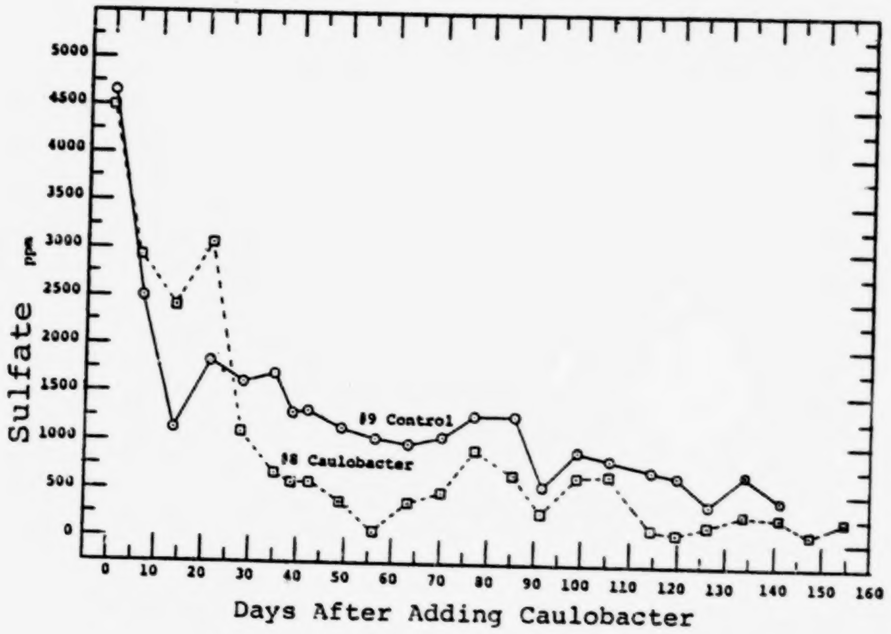


Figure 25 - Test 1

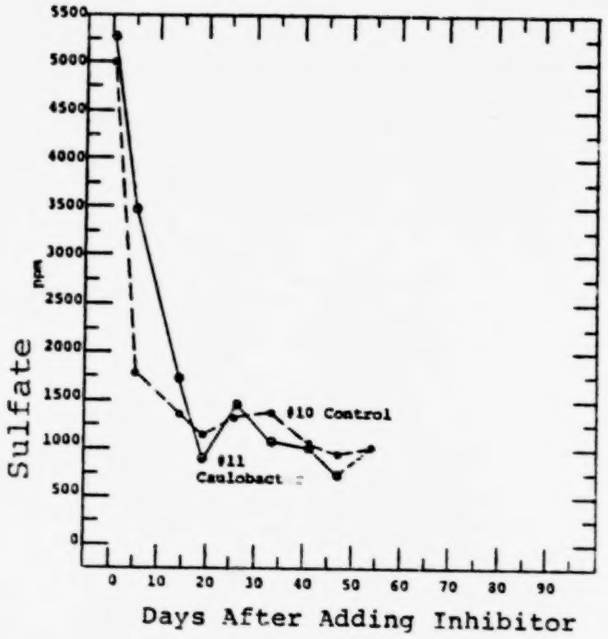


Figure 26 - Test 2

Effect of Caulobacter on Sulfate Production
by Robena Bacteria

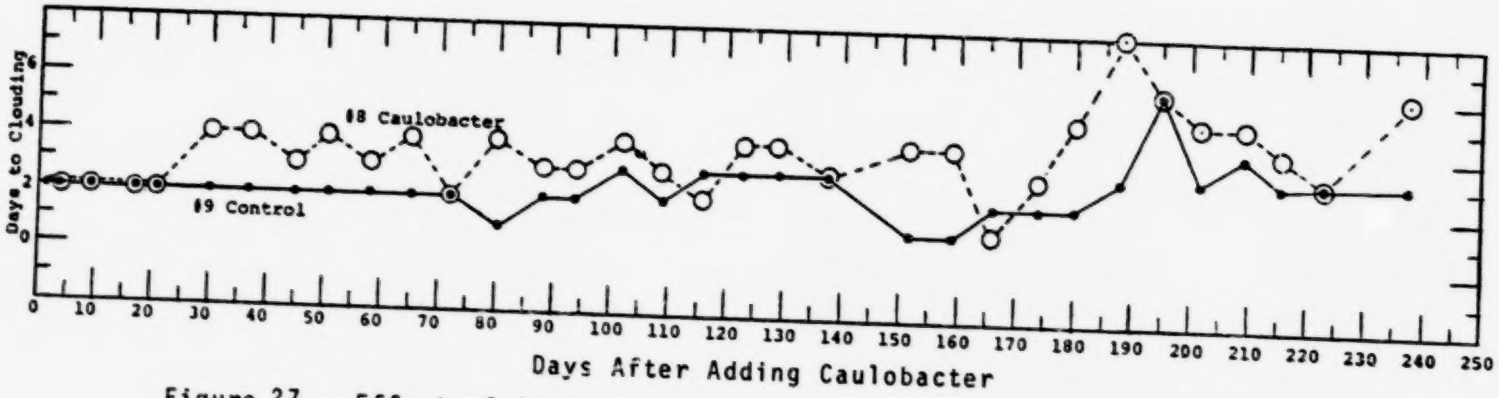


Figure 27 - Effect of Caulobacter on Oxidation Times of Robena Bacteria
Test 1

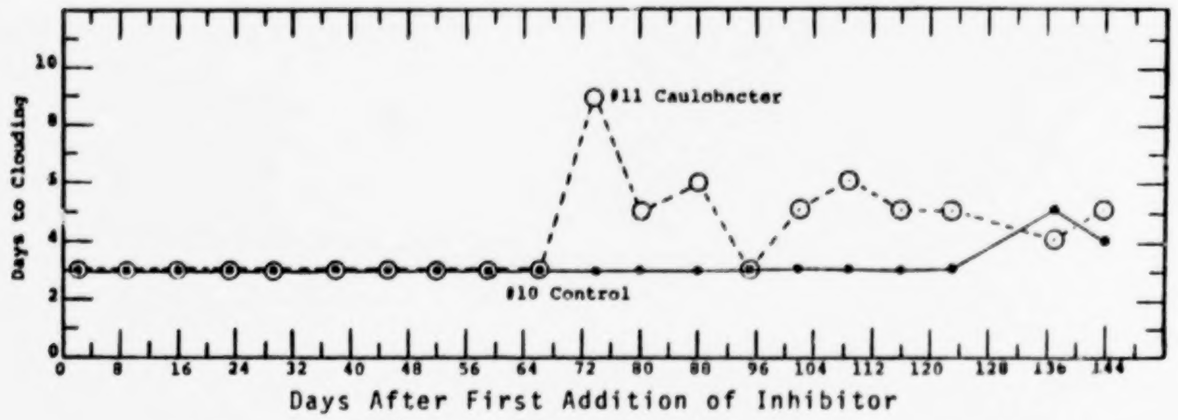


Figure 28 - Test 2
Effect of Caulobacter on Oxidation Times of Robena Bacteria

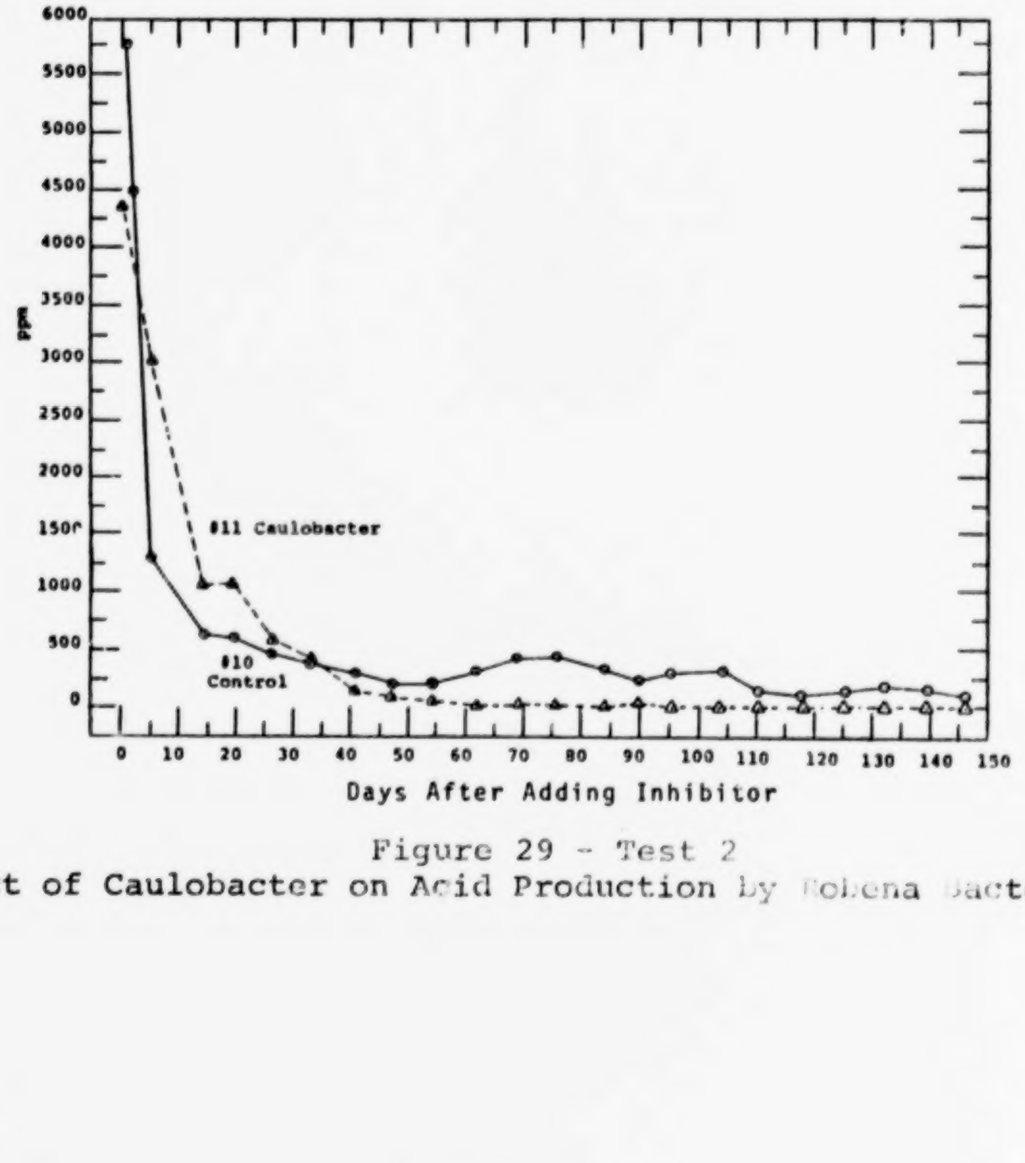


Figure 29 - Test 2
Effect of Caulobacter on Acid Production by Robena Bacteria

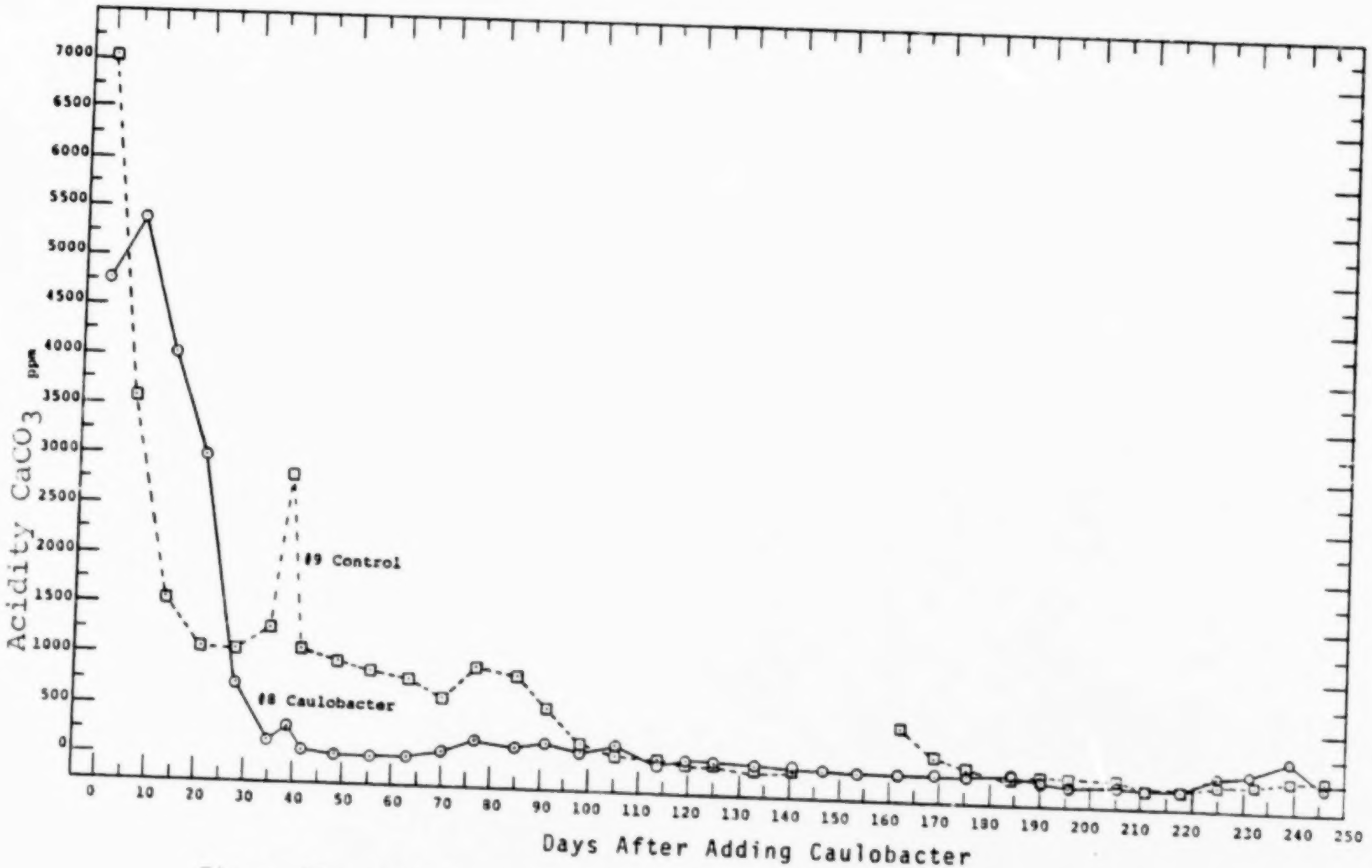


Figure 30 - Effect of Caulobacter on Acid Production by Robena Bacteria
Test 1

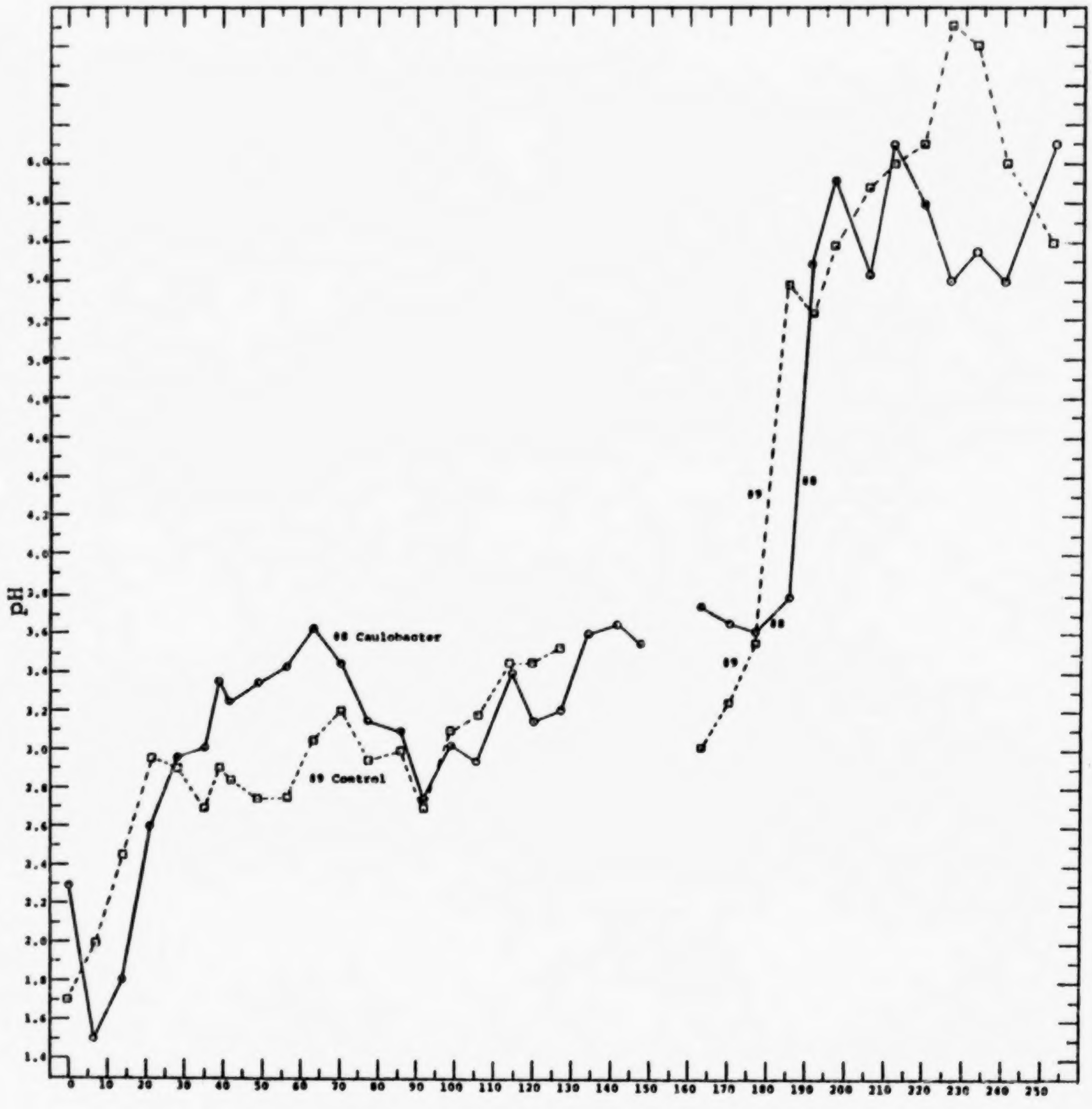


Figure 31 - Effect of Caulobacter in pH of Cultures of Robena Bacteria
Test 1

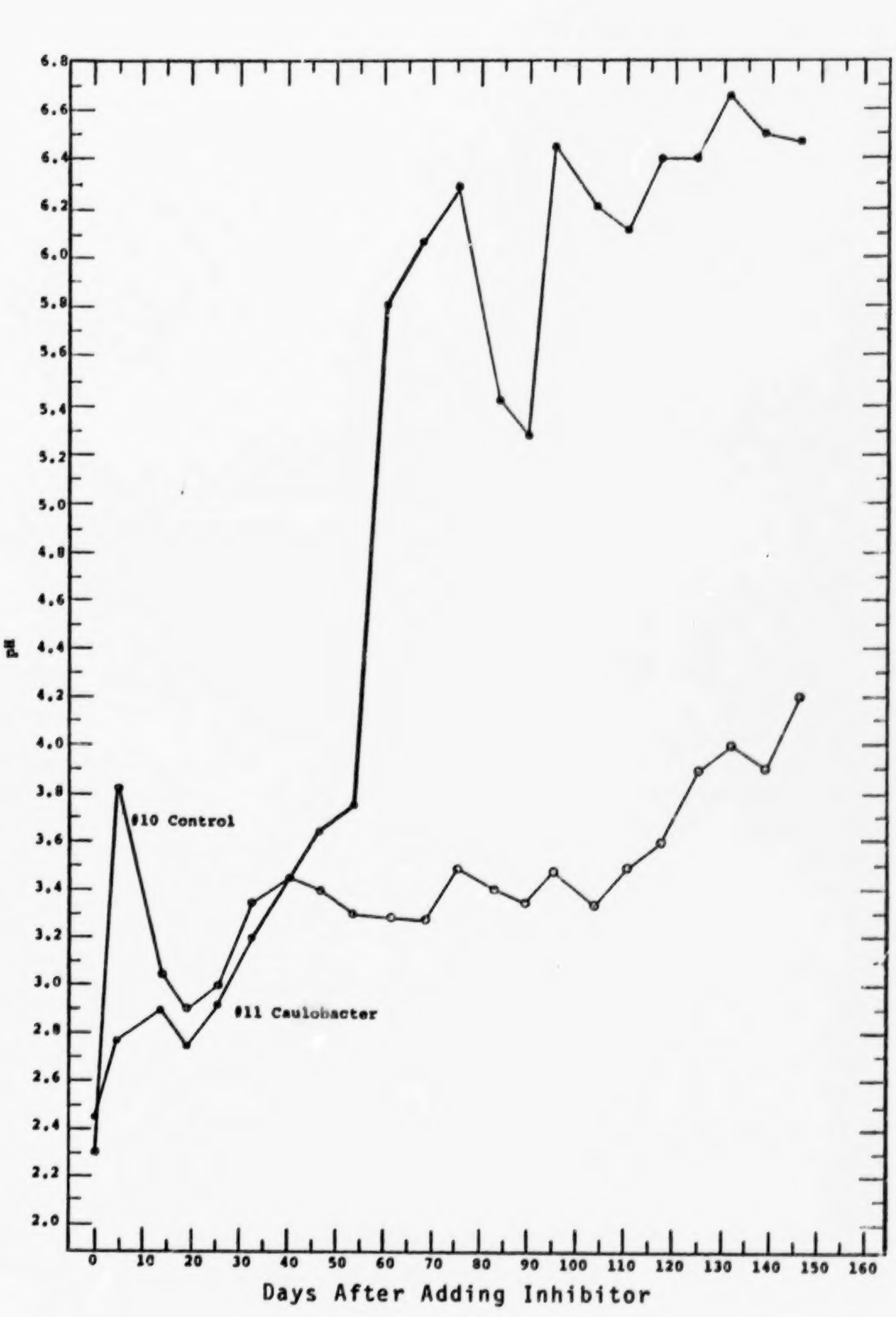


Figure 32 - Test 2

Effect of Caulobacter in pH of Cultures of Robena Bacteria hibition are given in Appendix 1.

A substitute for peptone must have been present in the flooded vertical coal systems as any initial peptone added with the Caulobacters would have been washed out in these flowing systems.

It seemed advisable to compare the number of viable Caulobacter cells from natural sources (Wildwood and McCormick waters) with those of the more concentrated McCormick and stock acid/ iron adapted strains.

Caulobacter Viability Studies

This work involved studies to determine the concentrations of viable cells of the adapted McCormick and stock Caulobacter strains. Comparisons were also made to determine the number of naturally occurring Caulobacter from McCormick and Wildwood mine area waters. Standard agar was used as a plating medium. Since a differential medium for Caulobacter does not exist;i.e., a medium which allows only Caulobacter to grow, accurate counts cannot be obtained from the mine area waters. However, estimates of the total number of viable cells can be made of a variety of microorganisms which can be grown on this plating medium. Many soil and water microorganisms are present in neutral mine waters which can grow on the standard medium. Fairly accurate Caulobacter counts can be obtained from the stock and adapted Caulobacter as these are pure cultures. The stock and McCormick strains of Caulobacters were grown in a synthetic mine drainage. Therefore, the same sterile mine drainages were used to prepare serial dilutions which were subsequently plated on standard agar. The Wildwood and McCormick waters were serially diluted in tap water with 0.01% peptone. This is commonly used to isolate Caulobacter from natural sources. The inhibitory waters were about one month old and filtrates from these waters had shown inhibitory plaques against Robena iron bacteria on iron agar plates. If natural sources are allowed to stand for any length of time, naturally occurring Caulobacters tend to increase in numbers. Poindexter, p-234 (1964) has stated that Caulobacter numbers increase with age in water supplies. These drainages, therefore, should contain Caulobacter and have shown some inhibitory power against iron bacteria.

Serial dilutions were made, aliquots plated and incubated. Counts were made on plates showing viable cells after three days of incubation. The stock and McCormick adapted Caulobacter were on the order of 10^{12} viable Caulobacter cells per ml. Two waters from Wildwood and two from McCormick all gave viability counts on the order of 10^7 cells per ml. It should be noted that this 10^7 number represents many types of microorganisms capable of growing on this medium. The number of viable Caulobacter colonies only would be lower.

The results show that the stock adapted Caulobacter strains are roughly 10^6 times greater in number than the naturally occurring strains. The 10^{12} cells of adapted Caulobacter is by no means an optimum number as greater cell yields can no doubt be obtained by using sedimented or supernatant cells. The results suggested that once optimum cell yields can be obtained, then it becomes feasible to inoculate mines with mass-produced packed cell cultures.

Initial Work with New Strains of Caulobacter

In accordance with the suggestions of the contract monitor, contract was made with Dr. Vincent Gerencser, consultant on Caulobacters. Dr. Gerencser suggested we test strains of Caulobacters which have lived in acid environments. These strains may easily adapt to acid/iron environments and possibly show some inhibition against the iron bacteria in their natural coal environment.

Four strains of Caulobacter vibroides were obtained from Dr. Gerencser. These strains were labeled C.V. 143, 144, 145 and 146. Although the source of these strains was a lake which has had a pH as low as 4.0, the strains were isolated at a later date when the pH of the lake water was 6.5. These strains appear to have a life cycle which permits them to live at different pH values. Since they have been isolated from waters having lower pH values, they appear to be good candidates for adaption to acid mine drainage.

Adaption of Caulobacters in Mine Drainage

The four species were stained and observed in the microscope for later comparison with the same strains adapted to an acid iron environment. The four strains appeared as typical stalked forms. The strains were transferred from the initial proprietary growth medium designed by Gerencser (1969) to the same medium adjusted to pH 7.0 and 4.0. This growth medium does not contain iron. Transfers were also made to a synthetic acid mine drainage without ferrous iron but adjusted to pH 3.9 using sulfuric acid. Additional transfers were made to synthetic acid mine drainage containing ferrous iron and adjusted to pH 3.6 with sulfuric acid. All transfers were made in duplicate in flasks which were then incubated while being shaken continuously to obtain optimum growth.

After six days, all flasks showed visible growth in

Gerencser's medium at both pH 7 and 4. One strain showed visible growth in synthetic acid mine drainage with and without ferrous iron. All four strains showed visible growth in liquid acid synthetic mine drainage, as evidenced by clouding of the medium. However, ferrous iron was not oxidized, indicating their growth was not at the expense of oxidizing iron. The nutrient source was iron medium A as described in Table 1. All strains were transferred 10 times in liquid acid synthetic mine drainage and continued to show good growth.

Aliquots from the Caulobacter vibroides (C.V.) series 143 through 146 grown in Gerencser's medium and also in synthetic acid mine drainage, were plated on standard agar. The C.V. colonies plated from Gerencer's medium were white, confluent and appeared to have a slime coating. The C.V. colonies plated from synthetic acid mine drainage were grey-white, raised, glistening and appeared smaller than the colonies obtained from Gerencser's medium. The acid/iron adapted C.V. series colonies appeared to be similar in appearance to the colonies obtained from acid/iron adapted stock and McCormick Caulobacters.

Static Testing Against Iron Bacteria

The four C.V. strains, after having been grown in synthetic iron medium A., were tested against Robena iron bacteria, also in synthetic A and I acid iron media as described on Table 1. A 1:1 ratio of Caulobacters to Robena iron bacteria were diluted 1:100 in the iron media. Three of the four C.V. strains caused a delay of two weeks in the oxidation of the Fe II by Robena iron bacteria in mine drainages I and A. The iron bacteria control tubes oxidized Fe II in two days, while the Fe II in the negative control tubes containing synthetic mine drainage only, remained unoxidized.

Testing the C.V. strains against Robena iron bacteria on iron agar plates showed that iron colonies were partially inhibited as indicated by clear areas on the plates.

The results showed that the four new strains of Caulobacters [C.V. series] were readily adaptable for growth under acid mine conditions and partially prevented iron bacteria from growing.

Some initial work was done in setting up vertical flooded systems for testing the newly obtained C.V. series strains of Caulobacters. However, work was stopped because of depletion of funds.

Flooded Horizontal System

To determine if Caulobacters could inhibit throughout a horizontal system when introduced only at the influent end, one liter of Caulobacter culture was introduced into one of the troughs used previously to test natural inhibitor. Results are shown in Table 18. The evidence, in general, is that there is effective inhibition and that the inhibition was moving downstream.

Ferrous iron levels at sample ports 3-2, 3-4 and 3-5 fell from the 30's to the range 0 to 10 ppm somewhat gradually, more rapidly in 3-2 and less so in 3-5, suggesting downstream travel of inhibitors. Somewhat similar conditions developed with sulfates, viabilities, acidities and total iron. pH failed to improve in port 3-5.

A second trough was readied for a replicate test, but this was not used because of depletion of funds.

TABLE 18 - EFFECTS OF CAULOBACTERS ALONG HORIZONTAL SYSTEMS

		#2 No inhibitor					#3 With Caulobacter				
	Days After Adding Inhibitor to #3	2-1•	2-2	2-4	2-5	2-9	3-1	3-2	3-4	3-5	3-9
Ferrous Iron (ppm)	-1	0	0.1	15	14	41	0	35	39	38	21
	6	0	2.5	33	2.8	101	0	15	111	40	108
	13	0	8	50	28	52	0.1	36	132	60	135
	20	0	1.8	40	7	70	0	16	130	0	200
	27	0	1.1	15	3.8	47	0	0	4.5	0	54
	34	0	1	30	7	31	0	0	13	11	63
	41	0	5	29	23	53	0	35	220	265	264
	48	0	0	0.2	0.2	6	0	0	60	21	41
	56	0	3	3	16	28	0	0	6	28	42
	62	0	8	29	6	65	0	0	0	26	60
	68	0	6	46	22	32	0	0	0	27	25
	76	0	15	25	10	16	0	0	0	10	--
Sulfates (ppm)	-1	1000	125	160	375	850	150	710	1100	875	1025
	6	125	250	425	475	1025	125	600	1000	625	1150
	13	150	175	400	600	1075	250	700	1000	600	1250
	20	125	100	425	550	1000	125	200	750	250	1475
	27	145	350	150	400	850	100	100	600	145	1000
	34	125	275	425	500	850	150	150	700	425	1650
	41	150	300	150	100	1125	140	275	1000	1025	1700
	48	60	30	90	175	600	90	100	725	275	1325
	56	175	175	50	40	625	100	125	250	250	1075
	62	125	425	265	300	850	125	50	175	125	1325
	68	125	275	125	125	710	100	140	100	125	1125
	76	120	100	250	300	410	100	100	150	225	1116
Viability (thousands of cells/ml)	0	2.5	30	0.8	30	8	10.9	30	30	30	9.5
	8	5	30	30	30	30	8.5	14.5	28	34	4.6
	15	3.3	30	21.5	30	30	2.8	18.5	22	78	240
	23	3	8	12	14	6	1.3	0.4	1.4	10.8	1.2
	29	2	4	17	20	4	1	0.1	1.6	2.3	5
	37	2	1	7	300	10	2.5	0.1	13	3.4	4
	43	3	35	16	80	15	4.5	0.7	20	35	20
	50	7	28	8	40	13	.5	0.1	0.5	0.3	2.8
	57	18	13	10	74	36	6.3	0.1	0.4	3.9	5.8
	64	2	12	11	26	10	10	0.4	0.5	4.3	2.5
	71	0.9	1.3	1.3	30	5	1	1	1	4	1
	79	2	70	32	300	105	1.8	1.8	2.1	2.8	30

TABLE 18 - EFFECTS OF CAULOBACTERS ALONG HORIZONTAL SYSTEMS (cont'd)

pH	-1	4.45	3.72	3.35	3.2	2.87	4.0	3.6	3.65	3.85	4.82
	6	3.5	2.65	2.5	2.42	2.15	3.62	3.3	3.05	3.1	2.85
	13	3.7	2.9	2.6	2.5	2.3	3.75	3.7	3.05	3.15	2.85
	20	3.72	3.1	2.8	2.65	2.4	5.9	3.9	3.05	3.15	3.0
	27	4.35	2.75	2.9	2.6	2.3	5.95	6.2	3.7	3.4	3.2
	34	3.7	2.7	2.6	2.55	2.3	3.95	6.3	5.1	3.1	2.95
	41	3.5	2.7	2.7	2.5	2.2	3.8	4.88	2.85	2.55	2.95
	48	3.7	2.7	2.9	2.6	2.4	4.2	3.78	3.25	2.9	3.15
	56	3.95	2.8	3.15	2.75	2.35	4.42	5.8	6.0	3.1	3.0
	62	4.95	2.6	2.75	2.6	2.25	3.95	5.3	6.2	3.0	3.1
	68	5.55	2.7	2.6	2.6	2.4	4.4	5.55	6.4	3.05	3.05
	76	5.45	2.95	2.8	2.6	2.45	5.75	6.05	6.8	3.35	3.10
Acidity (ppm)	-1	30	109	320	545	1385	55	355	375	395	190
	6	40	310	430	625	1695	55	205	515	375	820
	13	20	155	390	605	1530	20	130	485	310	745
	20	25	105	345	550	1615	-5	75	445	150	770
	27	20	250	160	490	1050	20	-5	70	60	360
	34	25	335	380	420	1040	20	-15	55	175	575
	41	45	255	255	485	1230	25	80	595	835	770
	48	35	215	160	365	720	15	14	30	255	365
	56	30	230	80	300	755	16	2.5	20	165	540
	62	14	410	235	300	1010	26	15	61	172	700
	68	4	295	305	435	855	10	6	-10	153	662
	76	12	150	230	420	700	3	1	16	116	550
Total Iron (ppm)	-1	5.2	12.2	47	97	271	7.8	90	106	109	53
	6	0	39	60	144	337	0	24	129	53	154
	13	1.8	16	61	104	305	1.6	38	154	68	202
	20	0.1	10	53	91	353	0	21	161	8	238
	27	0.2	36	24	90	228	0.1	0.2	12	31	70
	34	2	56	67	68	240	0.1	0.1	28	43	168
	41	2	42	48	97	323	3	41	256	314	351
	48	26	31	26	64	153	26	7	106	74	136
	56	1	40	9	57	164	3	5	14	36	184
	62	2	84	40	53	257	5	2	3	38	276
	68	0.5	55	63	70	190	1	1	6	35	281
	76	0	21	37	83	73	0	1	5	15	223

* 2-1, 2-2 etc refer to sample ports in numerical succession starting at influent.

BLANK PAGE

SECTION 13

STREPTOMYCES AS PRODUCERS OF ANTIBIOTIC INHIBITORS

Theoretical Approach

Microorganisms of the order Antinomycetales (especially Streptomyces which produce antibiotics) are found in the soil and could conceivably be present in neutral drainage. The waters from Wildwood and McCormick areas might contain antibiotic-producing bacteria and resultant antibiotics could kill iron bacteria. Samples of these waters were plated on Czapek's agar media but so many molds were present that any growth of the Streptomyces might have been obscured. A thorough literature search was not made to determine if the antibiotic-producing Streptomyces could produce antibiotics under stressed conditions as found in acid mine drainage, but a brief review of the bacteriological literature did not reveal any attempts to perform work of this nature.

It was proposed that antibiotic sensitivity disks first be obtained and tested against all strains of iron and sulfur bacteria. If effective against the iron bacteria, the microorganisms producing these antibiotics antagonistic to iron bacteria could be obtained and adapted to the environmental acid/iron conditions of iron bacteria. It was hoped that mutant or adapted strains of certain Streptomyces could be induced to grow in acid mine environments and continue to produce antibiotics which would kill off the iron and sulfur bacteria.

Antimicrobial Sensitivity Disk Testing

Various selective antibiotic disks were obtained and tested against the five strains of iron bacteria as shown in Table 19. The selective disks contained antibiotics produced by Streptomyces species which might conceivably be present in the soil of mine environments. However, a detailed study was not made to determine the pH of the various Streptomyces-producing antibiotics. Initial testing was done only to determine if any of the antibiotics inhibited the iron bacteria. Disks can be obtained in various dosage levels but, for our purposes, it was believed that disks should be tested at the lowest available concentration since future testing in mine areas would be limited to crude extracts of antibiotics to avoid high cost, or the use of antibiotic-producing strains of bacteria or possibly adaption of the same bacteria to acid mine conditions. In either case, the bacteria or antibiotic would not be as potent as highly purified antibiotics obtained from drug companies.

Plate testing was done by inoculating the solid iron agar in soft agar overlays. Each iron bacterium was tested separately.

Table 19 - Antibiotic Sensitivity Disk Testing

				Effectiveness on											
				Solid Iron Media(2)						Liquid Iron Media(3)					
Antiobiotic Disk	Micro-(1) gram	Produced By	Commercial Availibility	F. Sulfo	F. Ferro	T. Ferro	Karen	Robena	T.thio	F. Sulfo	F. Ferro	T. Ferro	Karen	Robena	T.thio
Novobiocin	5	S. Niveus S. Spheroides	Yes Yes	40	40	40	50	Clear	50	I	I	I	I	I	I
Methacycline	5	S. Rimosus		R	2	2	R	R	R	R	R	R	R	R	R
Dimethylchor-tetracycline	5			R	R	2	R	R	R	R	R	R	R	R	R
Neomycin	5	S. Fradie		R	R	R	R	R	R	R	R	R	R	R	R
Kanamycin (Kantrex)	5	S. Kanamyceticus		R	2	R	R	R	R	R	R	R	R	R	R
Lincomycin	2	S. Lincolnesis		R	R	R	R	R	R	R	R	R	R	R	R
Oleandomycin	2	S. antibio-ticus	Yes	1	1	1	NG	3	R	R	R	R	R	R	R
Tetracycline	5	S. species		2	1	2	NG	5	R	R	R	R	R	R	R
Chlorotetracyline (Aureomycin)	10	S. Aureo-faciens	Yes	2	2	2	2	2	30	R	R	R	R	R	I
Bacitracin A.	2U	B. Subtilis		1	R	2	2	2	R	R	R	R	R	R	R
Dihydro-streptomycin	2			R	R	R	R	R	R	R	R	R	R	R	R
Oxytetracycline (Terramycin)	10	S. Rimosus	Patented	2	2	2	NG	4	R	R	R	R	R	R	R
Streptomycin	2			R	R	1	NG	1	R	R	R	R	R	R	R
Nystatin	100U	S. noursei	Restricted	3	4	5	NG	7	R	R	R	R	R	R	R
Penicillin	2U			R	R	R	5	5	R	R	R	R	R	R	R

---

(1) These were the lowest available dosages which could be obtained.
(2) The zones of bacterial inhibition were measured from the edge of the disk and across the annulus of the clear area in millimeters. R - Bacteria were resistant. I - Iron did not become oxidized. NG - No bacterial growth.

After the agar hardened, each disk was placed onto the agar using sterile techniques. The plates were incubated until bacterial growth was evident and the zones of inhibition measured across the annulus of the clear zones.

Although not designed for use in liquid media, each type of disk was placed into 10 ml of synthetic iron media after first inoculating with iron bacteria. Each disk was tested individually against each strain of iron bacteria inoculated into synthetic I iron medium. Positive bacterial controls of each strain of iron bacteria, as well as negative controls, were included with each series of tubes tested. The tubes were incubated and observed daily for the appearance of oxidized ferrous iron.

Results are shown in Table 19. It was very clear that the antibiotic novobiocin was extremely effective at low dosages against all the strains of iron bacteria. On solid media, the zones of inhibition were very large and clear. No secondary satellite bacteria were seen. This indicated bactericidal action rather than just a bacteriostatic effect. Novobiocin also inhibited the bacteria in liquid media as evidenced by the tubes remaining clear for various lengths of time. The tube containing F. sulfo was clear for nine days after which it became partially oxidized and remained so for 41 days. F. ferro, Karen and Robena tubes remained perfectly clear for 41 days. The only strain against which novobiocin was not extremely effective in liquid media was T. ferro. The tube remained clear for ten days, after which it became oxidized as compared with the mere two-day inhibition of the control tubes.

After 41 days, the negative control tubes containing synthetic iron medium,but no bacteria,became partially oxidized. This indicated the beginning of chemical iron oxidation.

The excellent results obtained with novobiocin suggested that further studies be done with the antibiotic-producing microorganisms. Streptomyces niveus and Streptomyces spheroides. Novobiocin is an acidic antibiotic which consists of three moities: a sugar, a coumarin and a substituted phenol. Its action may be due to one of these compounds when used at pH 3.5 with the iron bacteria. However, rather than typing a chemical approach using various coumarins and phenolics, the bacteria producing this antibiotic were obtained and tested.

In summary, the antibiotics tested included novobiocin, methacycline, dimethychlortetracycline, neomycin, kanamycin, Kantrex, lincomycin, oleandomycin, tetracycline, chlorotetracycline, Aureomycin, bacitracin, dihydrostreptomycin, oxytetracycline, Terramycin, streptomycin, nystatin and penicillin. All antibiotics were tested as disks, except for bacitracin and penicillin, and are produced by various species of Streptomyces. The most effective antibiotic was novobiocin which killed all strains of iron bacteria, both in acid liquid synthetic iron medium and on iron agar plates. Two other antibiotics, oleandomycin and Aureomycin, showed inhibitory effects on plates but not in liquid.

An attempt was made to isolate the sulfur-oxidizing bacterium T. thiooxidans from flooded vertical coal pile systems. This was done since this bacterium is also involved in the oxidation of pyritic materials and an inhibitor might be found to be effective against it. Aliquots from all flooded vertical systems with Robena and Karen were placed into sulfur and thiosulfate broths at low dilution, 1:10. After three weeks of incubation, these cultures were diluted 1:100 with fresh nutrient broth. Higher dilutions had to be made to guard against false positive results due to chemical oxidation of sulfur. Oxidation of sulfur and thiosulfate solutions is a characteristic of the sulfur bacteria and is determined by clouding of the media with a drop in pH. The T. thio was isolated from flooded vertical system number one. Number one is the Karen bacterial control system containing bacteria from the Karen mine area. At the time the original aliquot was taken, the pH of this pile was 4.5 It was interesting to note that T. thio was not isolated from the other systems. It may be that either the pH was too high for the bacterium to be viable or this bacterium was never present after isolation on thiosulfate agar plates, antibiotic sensitivity tests were performed to determine if this bacterium was sensitive to any of the antibiotics.

Antibiotic disks were tested against the T. thiooxidans on solid thiosulfate agar plates and in a liquid thiosulfate medium. This organism grows as nearly transparent colonies on thiosulfate agar. Large number of colonies cloud the agar. In liquid thiosulfate, growth is characterized by a uniform turbidity; also, sulfur is precipitated with the medium becoming more acid.

The disks were aseptically added to heavy inocula of T. thio on thiosulfate agar and liquid medium. The results are shown on Table 19. Tubes and plates, along with control tubes, were then incubated until growth occurred in the control tubes and plates. It can readily be seen that only two antibiotics were effective against T. thio; these were novobiocin and Aureomycin. The sulfur bacterium was sensitive both on plates and in liquid media. These same two antibiotics were previously shown to be effective against the iron bacteria. Therefore, if the Streptomyces producing these antibiotics are active against the iron bacteria, they will also inhibit the sulfur bacteria.

Initial Testing of Antibiotic-producing Bacteria

Since some antibiotics were inhibitory to iron bacteria, the bacteria producing these antibiotics were obtained. Tests were conducted in order to determine if these special antibiotic-producing strains of Streptomyces could inhibit iron bacteria.

The antibiotic producers, S. niveus and S. spheroides (novobiocin), S. antibioticus (oleandomycin), and S. aureofaciens (Aureomycin), were each grown in 100 ml quantities of media commonly used to grow these bacteria, i.e., Czapek's, (Hesseltine, p-142 (1959)) obtained from Balitmore Biological Laboratories, and sporulation broth, American Type Culture Collection Catalogue, p-130 (1968). Sporulation broth is a complex organic liquid composed of yeast extract, beef extract, tryptose, glucose and traces of ferrous sulfate. The pH was 7.2. The Streptomyces species did not show early growth in Czapek's medium which is less complex than sporulation broth, and is composed of sucrose, sodium nitrate, dipotassium phosphate, magnesium sulfate, potassium chloride and ferrous sulfate. This medium has a pH of 7.3. Both fermentation liquids are about neutral in pH and contain sugars used for metabolism of heterotrophic bacteria. The Streptomyces were grown in flasks by shaking, using aeration to obtain better growth. After one week, good growth of all the Streptomyces species was obtained in sporulation medium but not in Czapek's nutrient solution, but after forty days good growth of the Streptomyces species was obtained in Czapek's solution.

After good growth was obtained, crude filtrates were prepared in order to remove debris. Filtrates were prepared by filtering the fermentation broths through Whatman filter paper. The filtrates were then tested against the iron bacteria within a few hours after the filtrates were prepared. The filtrates were diluted 1:100, 1:1000 and 1:10,000 in synthetic iron solution I as given in Table 1, then the strains of iron bacteria were added.

The results are shown in Table 20. The final dilution of filtrate in the synthetic iron was 1:100. Higher dilutions did not inhibit any of the iron bacteria. It was interesting to note that incomplete iron oxidation occurred in many tests and stayed at that point, never becoming completely oxidized. This is an infrequent occurrence, as in most cases incomplete bacterial oxidation is followed in one or two days by complete ferrous iron oxidations. It should be understood that the crude filtrates were diluted 1:100 in syntehtic iron media and were used against cultures of iron bacteria normally producing on the order of 10^7 to 10^8 viable cells per ml. In addition, the growth conditions for the Streptomyces were not optimal; i.e., special growth factors were not added as information about them is proprietary to the drug manufacturers. The results tend to show that even crude cultures of the effective antibiotic producing Streptomyces strains in-

TABLE 20 - TESTING ANTIBIOTIC PRODUCER PRODUCTS AGAINST IRON BACTERIA

Streptomyces species	Iron bacteria	Oxidation of ferrous iron in days	Oxidation of ferrous iron bacterial control tubes days[4]	Inhibitory effect
S. aureofaciens	F. sulfo	2	2	None
" "	F. ferro	2	2	None
" "	T. ferro	2	2	None
" "	Karen	4-partial[2] 5-complete[3]	2	Partial
" "	Robena	2	2	None
S. antibioticus	F. sulfo	10-partial[2] not complete in 21[3]	2	Good
" "	F. ferro	10	2	Good
" "	T. ferro	7	2	Fair
" "	Karen	14	2	Fair
" "	Robena	14-partial[2] 17-complete[3]	2	Good
S. niveus	F. sulfo	10-partial[2] not complete in 21[3]	2	Completely inhibitory
" "	F. ferro	5-partial[2] 21-complete[3]	2	Excellent
" "	T. ferro	6	2	Fair
" "	Karen	not oxidized in 21 days	2	Completely inhibitory
" "	Robena	5-partial[2] 21-complete[3]	2	Excellent
S. spheroides	F. sulfo	5-partial[2] 17-complete[3]	2	Excellent
" "	F. ferro	5-partial[2] 17-complete[3]	2	Excellent
" "	T. ferro	4-partial[2] 7-complete[3]	2	Fair
" "	Karen	not oxidized in 21 days	2	Completely inhibitory
" "	Robena	4-partial[2] 6-complete[3]	2	Fair

---

[2] In partial oxidation, the liquid portion of tube is clear and sediment is white (unoxidized iron) with a small amount of orange (oxidized) iron.

[3] In complete oxidation, the liquid becomes entirely orange and sediment is all orange.

[4] Oxidation results were obtained using aliquots grown in sporulation broth. Later aliquots from Czapek's broth showed oxidation times to be on the same order of magnitude.

hibited iron bacteria. However, it was not known if the antibiotic-producing Streptomyces bacteria produced antibiotics in their natural growth medium which subsequently inhibited the iron bacteria upon being transferred to an acid iron medium, or if the Streptomyces were growing and releasing antibiotic in the acid iron medium.

In order to determine if the Streptomyces were growing in the acid iron media, aliquots were removed from the inhibited iron bacteria tubes and plated on iron and Czapek's agars. No Streptomyces species were seen on the iron and Czapek's agars. Thus,it appeared the Streptomyces did not grow on iron medium.

Tests were made to determine if it was the Streptomyces bacteria, or their antibiotics products,which were responsible for inhibition. Aliquots were removed from the sporulation medium of the actively growing Streptomyces species. The aliquots were divided in half, with one portion added directly to acid synthetic mine drainage. The other portion was filtered, using Whatman paper, then millipore filtered for sterilization. The sterile filtrates were added to a second series of tubes containing acid synthetic mine drainage. Each of the strains of iron bacteria was added separately to the tubes. After incubation, it was found that both the filtered and unfiltered aliquots inhibited the iron bacteria. It appeared that it was the antibiotics rather than the bacteria responsible for inhibition. Additional tests were made in which Streptomyces aliquots were millipore filtered, the filter pad washed with sterile water, and the filter itself added to tubes of acidic synthetic mine drainage. The iron bacteria were added and the tubes incubated. It was found that the Streptomyces retained on the filters also inhibited the iron bacteria. However, the tubes did not cloud nor show any signs of bacterial growth indicating that the Streptomyces did not grow in the synthetic mine drainage. The conclusion appeared to be that the growth and antibiotic products produced by the Streptomyces are closely bound and it is difficult to separate the Streptomyces bacteria from their antibiotic products. In spite of the fact that the millipore-retained Streptomyces bacteria inhibited iron bacteria, no growth of the Streptomyces was observed in tubes of synthetic mine drainage; therefore, the Streptomyces did not adapt to the iron media under those conditions. It would be correct then to state that antibiotics produced by certain Streptomyces species inhibited iron bacteria in synthetic acid mine drainage; however, the species will not grow in acid mine drainage.

Partial Adaption of Antibiotic-producing Streptomyces

Since adaption of the Streptomyces species did not occur upon direct transfer to synthetic acid mine drainage, attempts were made to gradually adapt these species to coal mine environments. Initial attempts involved gradually decreasing the sugar content and increasing the iron and acid concentration. Sporulation and synthetic iron media were mixed in equal quantities. The final pH was 5.4 as compared with 6.8 of the sporulation medium alone. Aliquots from the liquid sporulation medium showing good growth of the Streptomyces species were transferred to the mixed solution. This was done to determine if the antibiotic-producers could become gradually adapted to acid iron conditions. The flasks were shaken and aerated as before. After seven days, no visible growth was noticed in the flasks. However, filtrates were prepared and tested against the iron bacteria as before. The filtrates did not inhibit the iron bacteria.

The flasks were allowed to incubate for three weeks, then retested against iron bacteria. At this time, growth was seen in the flask containing S. aureofaciens, but no growth was observed in flasks containing S. antibioticus, S. niveus and S. spheroides. The S. aureofaciens aliquot inhibited Karen iron bacteria for four days beyond the control tube, but no other species of iron bacteria were inhibited. The other antibiotic producers did not inhibit any strain of iron bacteria.

It had been previously reported that Streptomyces did not grow well in Czapek's medium. However, it was found that growth did occur after an incubation time of 40 days. Tests conducted with aliquots from flasks of the various Streptomyces species in this medium showed that inhibition of iron bacteria was of the same order of magnitude as those from the original sporulation medium. Since the Streptomyces antibiotics inhibited iron bacteria when transferred from Czapek's solution to synthetic mine drainage, attempts were made to adapt the Streptomyces species to coal mine environments. In an attempt at adaption, aliquots of the various species of Streptomyces, actively growing in Czapek's nutrient, were transferred to coal and mine drainage in flasks. Coal (10 g) and pyrite (2 g) were added to each flask containing 100 ml of synthetic I mine drainage, (Table 1). Ferrous sulfate was not added to the medium since it was felt that coal and pyrite would supply the iron and sulfur needed. An additional modification was made by adding one ml of 10 percent sucrose to the medium as an added energy source required by the Streptomyces. The pH of the medium was adjusted to pH 3.9 using sulfuric acid. The flasks were aerated and shaken as before. After six days, growth appeared on the liquid surface of each flask. Aliquots were transferred to Czapek's agar. In all instances, the Streptomyces grew on the agar as bright yellow colonies; the S. spheroides colonies were lytic showing clear areas around each colony. It is evident that variants have occurred as these colonies differ markedly from their normal chalky-white appearance. The characteristic earthy odor, although not as strong as normal,

Table 21 - Inhibitory Effect of Coal-Grown Streptomyces (Sucrose Added) (16 Days)

Streptomyces Species	Iron Bacteria	Degree of Ferrous Iron Oxidation (Days)	Oxidation of Ferrous Iron-Bacterial Control Tubes (Days)	Inhibitory Effect
S. spheroides	F. sulfo	2 complete[2] - 11 partial[1] - 22 partial	2	Greater with time
S. spheroides	F. ferro	4 partial - 22 partial	2	Greater with time
S. spheroides	T. ferro	4 partial - 11 clear[3] - 22 partial	2	Almost complete with time
S. spheroides	Karen	4 partial - 17 complete	2	Not increased with time, effect is partial
S. spheroides	Robena	4 complete	2	Slight partial inhibition, not increased with time
S. niveus	F. sulfo	4 partial - 22 partial	2	Greater with time
S. niveus	F. ferro	4 partial - 22 partial	2	Greater with time
S. niveus	T. ferro	4 partial - 11 complete	2	Partial inhibition, not increased with time
S. niveus	Karen	4 partial - 11 complete	2	Same as above
S. niveus	Robena	4 partial - 22 partial	2	Greater with time

[1] In partial oxidation, the lower portion of the liquid is clear, sediment is white (unoxidized iron), and upper portion is orange (oxidized) iron.

[2] Complete oxidation - the liquid becomes entirely orange and sediment is entirely orange.

[3] In this test, iron became partially oxidized and then cleared at 11 days, followed by partial oxidation at 18 days.

was still present. It is apparent that these Streptomyces strains have adapted to a new environment.

Aliquots were tested as before against the iron bacteria. However, no inhibition occurred with the exception of S. aureofaciens which inhibited Karen iron bacteria only. It appears that while the Streptomyces species adapted to acid mine environments, the ability to produce effective antibiotics was impaired.

Growth of the Streptomyces in flasks containing coal in mine drainage was allowed to continue for 16 days, then aliquots retested against the iron bacteria in liquid synthetic acid mine drainage. In this group of tests a 1:5 ratio of iron bacteria to Streptomyces bacteria was used. The Streptomyces aliquots were increased in order to increase the chances of demonstrating antibacterial action against the iron bacteria.

The results shown on Table 21 indicate some very interesting phenomena; some of the cultures passed from complete oxidation to partial or almost complete clearing with time. The tubes which showed bottom-clearing were marked and it was found that the bottom cleared area tended to rise upward in all tubes. It is evident that this marked clearing is due to the growth of the Streptomyces species as the liquid assumed a milky, cloudy appearance caused by bacterial growth. This reversal of the orange color of oxidized iron had not occurred before. It was originally thought that the orange color of oxidized iron was irreversible, since it is a chemical change resulting from the growth of the iron bacteria. The other two species of antibiotic-producers were not included in this test, as previous results showed them to be weaker. In summary, larger aliquots of some Streptomyces, grown in flasks containing coal, sucrose and acid drainage, showed inhibitory effects against the iron bacteria. It might be that Fe III was reduced by pyrite catalytically.

Growth of the Streptomyces species in coal flasks was allowed to continue for an additional five days for a total of 21 days, then aliquots retested against iron bacteria at a 1:1 ratio . The results are shown in Table 22. It can be seen that S. niveus and S. antibioticus show fairly good inhibition of the iron bacteria. It is evident from the results that the Streptomyces require a good deal of time to become adapted to new growth conditions as no inhibition was shown in the early stages of growth (six days); but fairly good inhibition was evident at 21 days. Increasing the ratio of Streptomyces growth aliquot over the iron bacteria also showed a good inhibitory effect when Streptomyces cultures were grown for 16 days.

Results indicated that some types of Streptomyces partially

Table 22 - Inhibitory Effect of Coal-Grown Streptomyces (Sucrose Added) (21 Days)

Streptomyces Species	Iron Bacteria	Degree of Ferrous Iron Oxidation (Days)	Oxidation of Ferrous Iron-Bacterial Control Tubes (Days)	Inhibitory Effect
S. spheroides	F. sulfo	3 partial[1]- 6 complete[2]	2	Low inhibitory effect
S. spheroides	F. ferro	" "	2	" " "
S. spheroides	T. ferro	" "	2	" " "
S. spheroides	Karen	" "	2	" " "
S. spheroides	Robena	" "	2	" " "
S. niveus	F. sulfo	2 partial - 14 complete	2	Partial inhibition
S. niveus	F. ferro	" "	2	" "
S. niveus	T. ferro	" "	2	" "
S. niveus	Karen	" "	2	" "
S. niveus	Robena	" "	2	" "
S. antibioticus	F. sulfo	10 partial - 16 complete	2	Partial inhibition
S. antibioticus	F. ferro	8 partial - 14 complete	2	" "
S. antibioticus	T. ferro	6 partial - 14 complete	2	" "
S. antibioticus	Karen	6 partial - 16 complete	2	" "
S. antibioticus	Robena	8 partial - 14 complete	2	" "
S. aureofaciens	F. sulfo	2 partial - 3 complete	2	Low inhibitory effect
S. aureofaciens	F. ferro	2 partial - 3 complete	2	" " "
S. aureofaciens	T. ferro	2 partial - 6 complete	2	" " "
S. aureofaciens	Karen	2 partial - 6 complete	2	" " "
S. aureofaciens	Robena	2 partial - 3 complete	2	" " "

[1] Partial oxidation - the liquid portion is partially clear, and sediment is white (unoxidized iron).

[2] Complete oxidation - the liquid becomes entirely orange and sediment is entirely orange.

inhibited iron bacteria for 22 days in liquid synthetic acid mine drainage. The streptomyces were grown in flasks containing coal, pyrite and synthetic mine drainage with sucrose added as an additional supplement for growth. The inhibitory action was clearly shown when each antibiotic-producer was tested separately against each strain of iron bacteria in synthetic acid mine drainage without additional supplements. Test tubes showed an initial bottom clearing which gradually increased with time until 40 days when tests were terminated. The positive bacterial controls became completely oxidized in two days and remained oxidized until tests were stopped. Negative control tubes remained clear throughout the test. The tubes containing Streptomyces and iron bacteria cleared at the bottom first, then gradually the clearing of the orange color of oxidized iron was replaced by a milky, cloudy appearance. The tube contents became clear of oxidized iron except for iron which had adhered to the glass above the liquid surfaces. Color photographs were taken when tests were terminated and sent to certain representatives of our sponsors in this program. In addition, pH tests done at this time showed the inhibited tubes averaged about 2.8 as compared with 2.5 of positive bacterial control tubes. This indicated that iron was reduced and was in some way connected with the interaction of Streptomyces and/or antibiotic products with the iron bacteria. Tests conducted at a later time to help clarify these points are described below.

In order to determine if the inhibited tubes contained actively growing Streptomyces, aliquots were aseptically removed and plated on Czapek's agar. After incubation, yellow colonies of the adapted Streptomyces types were subsequently isolated. Tests were also made, to be described in a later part of this report, showing that iron bacteria do not grow on Czapek's agar nor do Streptomyces oxidize iron.

In summary, results indicate that certain types of Streptomyces will partially adapt to acid mine drainage if some sucrose is present. These adapted strains inhibit iron bacteria in acid mine drainage for varying periods of time. Methods of obtaining full adaptation without sucrose are described below.

Full Adaptation of Streptomyces

Studies were made to determine if the various strains of Streptomyces could fully adapt to acid/iron conditions, and continue to produce effective antibiotics against the iron bacteria. Aliquots from the four strains of Streptomyces, growing in coal, pyrite, sucrose and mine drainage, were transferred to fresh sterilized flasks of coal, pyrite and acid mine drainage without sucrose. These flasks, plain and irradiated, were aerated and shaken. At various time intervals, aliquots were removed and tested against all strains of iron bacteria in tubes of synthetic mine drainage. Aliquots tested after seven days of growth were negative for inhibitory powers.

Growth of the Streptomyces was allowed to continue for an additional ten days, or a total time of 17 days. Aliquots were then tested against iron bacteria at a 5:1 Streptomyces/iron bacteria ratio. Streptomyces aliquots were removed and additional tests made after total incubation times of 25 and 35 days at the same ratio. The results are shown on Tables 23, 24, and 25. It can be seen that only *S. aureofaciens* inhibited the iron bacteria regardless of the age of the Streptomyces culture. The *S. spheroides*, *S. niveus* and *S. antibioticus* had previously shown inhibitory action when grown in pyrite, coal, drainage and sucrose. When adapted to an autotrophic environment of coal, pyrite and acid drainage, the inhibitory action of these three species appeared to have been lost. Aliquots from Streptomyces coal flasks were placed onto Czapek's agar and incubated. The yellow colonies of adapted Streptomyces were isolated from all flasks. Therefore, the various Streptomyces strains have adapted to, or survived in the coal environment as evidenced by the growth on plates. However, some of these species lost their ability to produce effective antibiotics against the iron bacteria.

In summary, it appears that *S. aureofaciens* fully adapted to an acid coal environment and produced antibiotics effective against the iron bacteria at a 5:1 Streptomyces iron bacteria ratio. The other Streptomyces species will grow under full acid/iron conditions without supplements, but appear to have lost other antibiotic powers. It remains to be seen what effect the antibiotic producers would have in coal pile containers, especially on acid production and on iron oxidation.

Small flask cultures (200 ml) of *S. aureofaciens* and *S. spheroides* were used to seed 5 liter quantities of synthetic acid mine drainage containing coal and acidified pyrite. Culturing was done in large batch quantities to determine if better growth of the Streptomyces would occur. The nutrient materials were added to sterile, 2 gallon containers fitted with sterile glass fritted bubblers. Air intake and outlet lines were fitted with sterile millipore air filters

The seed cultures were added, air supply started and the containers shaken continuously. After 6 days of incubation excellent growth of the two antibiotic-producing Streptomyces speceis were obtained. These were then used to test their effects on flooded upward-flowing vertical acid-producing systems as described elsewhere.

Table 23 - Inhibitory Effect of Coal-Grown Streptomyces (No Additives) 17 Days

Streptomyces Species	Iron Bacteria	Degree of Ferrous Iron[1] Oxidation (Days)	Inhibitory Effect
S. spheroides	F. sulfo	2 complete	None
S. spheroides	F. ferro	2 complete	"
S. spheroides	T. ferro	2 complete	"
S. spheroides	Karen	2 complete	"
S. spheroides	Robena	2 complete	"
S. niveus	F. sulfo	2 complete	None
S. niveus	F. ferro	2 complete	"
S. niveus	T. ferro	2 complete	"
S. niveus	Karen	2 complete	"
S. niveus	Robena	2 complete	"
S. antibioticus	F. sulfo	2 complete	None
S. antibioticus	F. ferro	2 complete	"
S. antibioticus	T. ferro	2 complete	"
S. antibioticus	Karen	2 complete	"
S. antibioticus	Robena	2 complete	"
S. aureofaciens	F. sulfo	3 partial - 17 complete	Partial inhibition
S. aureofaciens	F. ferro	3 partial - 17 complete	" "
S. aureofaciens	T. ferro	3 partial - 17 complete	" "
S. aureofaciens	Karen	6 complete	" "
S. aureofaciens	Robena	6 partial - 17 complete	" "

[1] Partial oxidation - The liquid portion is partially clear, and sediment is white ferrous iron.
Complete oxidation - The liquid becomes entirely orange and sediment is entirely orange.
Control tubes containing no Streptomyces were completely oxidized in two days.

Table 24 - Inhibitory Effect of Coal-Grown Streptomyces (No Additives) 25 Days

Streptomyces Species	Iron Bacteria	Degree of Ferrous Iron(1) Oxidation (Days)	Inhibitory Effect
S. spheroides	F. sulfo	2 complete	None
S. spheroides	F. ferro	2 complete	"
S. spheroides	T. ferro	2 complete	"
S. spheroides	Karen	6 complete	"
S. spheroides	Robena	2 complete	"
S. niveus	F. sulfo	2 complete	None
S. niveus	F. ferro	2 complete	"
S. niveus	T. ferro	2 complete	"
S. niveus	Karen	2 complete	"
S. niveus	Robena	2 complete	"
S. antibioticus	F. sulfo	2 complete	None
S. antibioticus	F. ferro	2 complete	"
S. antibioticus	T. ferro	2 complete	"
S. antibioticus	Karen	6 complete	Partial inhibition
S. antibioticus	Robena	2 complete	" "
S. aureofaciens	F. sulfo	6 partial - 9 partial(2)	Partial inhibition
S. aureofaciens	F. ferro	6 partial - 9 partial(2)	" "
S. aureofaciens	T. ferro	6 partial - 9 partial(2)	" "
S. aureofaciens	Karen	6 complete	Low inhibitory effect
S. aureofaciens	Robena	6 partial - 9 partial(2)	Partial inhibition

(1) Partial oxidation - The liquid portion is partially clear, and sediment is white (unoxidized iron).
Complete oxidation - The liquid becomes entirely orange and sediment is entirely orange.
Control tubes containing no Streptomyces were completely oxidized in two days.

(2) This partial inhibition appeared to be stable at this point when chemistries were done.

Table 25 - Inhibitory Effect of Coal-Grown Streptomyces (No Additives) 35 Days

Streptomyces Species	Iron Bacteria	Degree of Ferrous Iron(1) Oxidation (Days)	Inhibitory Effect
S. spheroides	F. sulfo	2 complete	None
S. spheroides	F. ferro	2 complete	"
S. spheroides	T. ferro	2 complete	"
S. spheroides	Karen	2 complete	"
S. spheroides	Robena	2 complete	"
S. niveus	F. sulfo	2 complete	None
S. niveus	F. ferro	2 complete	"
S. niveus	T. ferro	2 complete	"
S. niveus	Karen	2 complete	"
S. niveus	Robena	2 complete	"
S. antibioticus	F. sulfo	2 complete	None
S. antibioticus	F. ferro	2 complete	"
S. antibioticus	T. ferro	2 complete	"
S. antibioticus	Karen	2 complete	"
S. antibioticus	Robena	2 complete	"
S. aureofaciens	F. sulfo	4 complete-6 partial-10 partial	Partial inhibition
S. aureofaciens	F. ferro	4 complete-6 partial-10 partial	" "
S. aureofaciens	T. ferro	4 complete-6 partial-10 partial	" "
S. aureofaciens	Karen	4 complete-6 partial-10 partial	" "
S. aureofaciens	Robena	4 complete-6 partial-10 partial	" "

(1) Partial oxidation - The liquid portion is partially clear, and sediment is white (unoxidized iron).

Complete oxidation - The liquid becomes entirely orange and sediment is entirely orange.

Control tubes containing no Streptomyces were completely oxidized in two days.

Tests to Determine if Streptomyces Oxidized Iron

Streptomyces colonies from all stages of partial and fully adapted growth were isolated on Czapek's agar, then picked and inoculated into test tubes containing synthetic acid mine drainage. After incubation, tube contents became cloudy with growth, but iron was never oxidized. These yellow colonies of adapted Streptomyces will not oxidize iron. In addition, all strains of iron bacteria were placed on Czapek"s agar to determine if they would grow and be mistaken for Streptomyces. It was found that iron bacteria would not grow on Czapek's agar.

Chemical Analyses of Streptomyces-Inhibited Tubes

It was shown previously that some Streptomyces species partially blocked ferrous iron oxidation in test tube studies. It was observed that some Streptomyces species cleared oxidized iron in nutrient synthetic acid-mine drainage in test tubes. In some cases, the iron bacteria oxidized iron, but this was followed by subsequent clearing from the bottom of the tubes upward. This clearing was caused by the action of the Streptomyces (or by-products). It was not known if a chemical or antibiotic produced by the Streptomyces species actually reduced iron.

In order to better define the chemical nature of partial iron bacteria inhibition, the Streptomyces and iron bacteria tubes were analyzed after 9 days of partial inhibition. These were compared with a positive iron bacterial control tube. The results are shown on Table 26. It can be readily seen that the ratio of ferrous to ferric iron has been changed. The tube containing S. aureofaciens and Robena iron bacteria showed that about 55 percent of the total iron was found in the ferrous state. The bacterial control tube shows only about 2% of the iron in the ferrous state. The other analytical results showed very little change.

Testing Streptomyces for Presence of Iron-Reducing Substances

A series of tests was set up to determine if the Streptomyces produced an iron-reducing substance. A ferric sulfate solution was prepared, sterilized by filtration and added to nutrient synthetic acid mine drainage in the same quantity as ferrous iron is added to prepare the synthetic acid iron solution normally used to test for the presence of iron oxidation. Test tubes were set up consisting of five series of 3 tests each. Ten ml of acid ferric iron solution was added to 1 tube of each series; a 0.5 ml quantity of S. niveus was added to the second tube of each series. A 0.5 ml quantity of non-oxidized synthetic ferrous iron solution was added to the third control tube of each series to compensate for the addition of 0.5 ml quantities of Streptomyces grown in synthetic mine drainage containing coal. One additional tube

Table 26 - Analyses of Streptomyces-Inhibited Tubes

Sample Designation	Fe^{++} ppm	Fe^{+++} ppm	Total Iron ppm	Sulfates ppm	pH	Total Acid ppm
S. aureofaciens vs F. sulfo	118	208	326	1650	2.52	1025
S. aureofaciens vs F. ferro	5	385	390	1630	2.80	1018
S. aureofaciens vs T. ferro	32	333	365	1425	2.90	1025
S. aureofaciens vs Robena	208	169	377	1850	2.95	1038
Pos. Control - Robena	8	372	380	1400	2.80	1062

was set up containing the ferric iron solution with nothing else added. Each set of three tubes was analyzed at one time with each series being analyzed one week apart. Each set was discarded after analysis to avoid contamination. The final pH of the ferric acid iron solution was 2.5 which is lower than the normally used ferrous acid iron solution (3.6).

The results shown in Table 27 indicate little change in the chemical analyses, except for unexplained variation in sulfates. It is evident that an iron-reducing agent is not present under these test conditions. There was no indication that the Streptomyces species were growing in the ferric iron synthetic drainage media. It appears that the clearing caused by Streptomyces is possibly some interaction of Streptomyces and iron bacteria on ferric iron.

Microscopic Studies of Adapted Streptomyces

A study was made on *S. aureofaciens* at various stages of adaption to roughly determine if any changes occurred during adaption. This was done by placing small aliquots on glass slides, staining, and observing the structure in the microscope. All slides were compared with the original culture obtained from the American Type Culture Collection. The appearance of the Streptomyces was the same with coal-adapted Streptomyces, the yellow colonies on Czapek's agar taken from coal adapted Streptomyces and from Strpetomyces growing in a tube of liquid synthetic mine drainage. The appearance of each of these samples was the same as the original stock culture. The colonies on plates differed as the original stock culture growing on Czapek's agar had a white powdery appearance, while adapted coal colonies were yellow on Czapek's agar. However, the microscopic appearance of all cultures were the same. Thus, we can be fairly certain thea the *S. aureofaciens* has adapted to a coal environment. Further testing in flowing streams through coal piles made to determine if the various Streptomyces strains will continue to produce antibiotics effective against the iron and sulfur bacteria is described below.

S. aureofaciens appears to be a good candidate for an inhibitor as it produces chlorotetracyclene, an amphoteric antibiotic compound continaing both nitrogen and non-ionic chlorine active against various bacteria. It also produces tetracycline in a chlorine-poor environment.

Streptomyces in Flooded Upward Flowing Vertical Systems

Two of three containers with Robena bacteria producing acid were each inoculated with 100 ml of *Streptomyces aureofaciens* cultures and two of another group of three were inoculated with the same volume of *S. spheroides*. The effluents were monitored regular-

Table 27 - Testing for Presence of Streptomyces-Induced Iron Reduction

Sample	Time of analyses	Fe^{++} ppm	Fe^{+++} ppm	Total iron-ppm	Acidity ppm	Sulfates ppm	pH
Fe^{+++} in syn I	Immediate	11(1)	810(2)	810	2863	3875	2.25
S. spheroides	Immediate	10	675	675	1863	2125	2.45
S. niveus	Immediate	9	622	622	1869	2900	2.50
Control	Immediate	39	640	670	1856	2250	2.45
S. spheroides	1 week	14	650	650	2113	3125	2.45
S. niveus	1 week	14	655	655	1969	3500	2.50
Control	1 week	41	640	705	2019	3550	2.50
S. spheroides	2nd week	10	610	610	2083	2750	2.30
S. niveus	2nd week	12	628	628	2125	2250	2.35
Control	2nd week	34	645	665	2125	2250	2.32
S. spheroides	3rd week	12	618	618	2225	4200	2.25
S. niveus	3rd week	14	618	618	2275	4200	2.30
Control	3rd week	38	640	675	2233	4000	2.35
S. spheroides	4th week	15	590	590	2200	3125	2.15
S. niveus	4th week	18	600	600	2275	3250	2.25
Control	4th week	40	600	630	2250	3500	2.15

(1) This number is believed to be blank due to absorption of ferric sulfate color at this wave length.

(2) These numbers are approximate since low reading obtained from ferrous iron were blank.

ly for pH and total acidity. Results are shown in Tables 28 through 30 and in Figures 33 through 35.

Table 28 - Viability of Robena Bacteria in Systems Inoculated with S. Aureofaciens

Cells per Milliliter

Days After Inoculation	#1 Control	#2 Test	#3 Test
0	1,000	300,000	2,000
7	9,000	8,300	2,300
14	4,000	1,000	100
21	4,000	400	1,000

Table 29 - Viability of Robena Bacteria in Systems Inoculated with S. Spheroides

Cells per Milliliter

Days After Inoculation	#1 Control	#2 Test	#3 Test
0	3,000	9,000	7,000
7	14,000	1,100	100
14	700	12,000	2,300
21	1,000	2,500	1,400

It can be seen that S. aureofaciens has some inhibitory effect on both the chemistry and microbiology of the systems but that S. spheroides has no such effect. Viabilities in systems with S. aureofaciens fell off significantly, acid production was decreased and the mean pH of the two test systems along with data on other systems generally showed a rise. A statistical analysis of these data along with data on other systems is given in Appendix 1.

Table 30 - Test Data on Streptomyces as Inhibitor

Days After Adding S. spheroides	pH			Acidity (ppm)		
	No. 4 Control	No. 5 Test	No. 6 Test	No. 4 Control	No. 5 Test	No. 6 Test
	2.80	2.95	2.89	320	212	610
-3	2.80	2.95	3.10	640	370	280
5	2.92	3.05	3.10	520	380	380
11	2.90	2.92	2.90	360	420	580
17	2.90	2.70	2.80	500	400	380
25	2.90	2.60	2.70	350	770	400
32	2.90	2.70	2.80	210	890	510
39	2.90	2.70	2.60	400	820	480
46	2.80	3.10	2.90	480	760	450
53	2.80	2.90	2.70	420	620	450
60	--	2.90	2.90	1050	390	390
74	3.00	3.00	2.90	550	150	140
81	2.80	2.90	2.80	320	350	410

	Viabilities (thousands of cells/ml)			Oxidation Times (days to clouding)		
-1	3	9	7	---	---	---
7	14	1.1	<0.1	4	4	3
14	0.7	12	2.3	3	3	5
21	1.0	2.5	1.4	4	4	3
28	1.6	1.1	0.8	4	3	3
35	74	4.8	3.7	3	3	3
42	2.8	0.8	0.1	3	3	3
49	5	0.7	4	3	3	3
56	<0.1	<0.1	<0.1	6	8	8
63	10	0.1	0.9	3	4	4
70	--	--	--	4	5	4
77	9	0.5	3	3	4	4

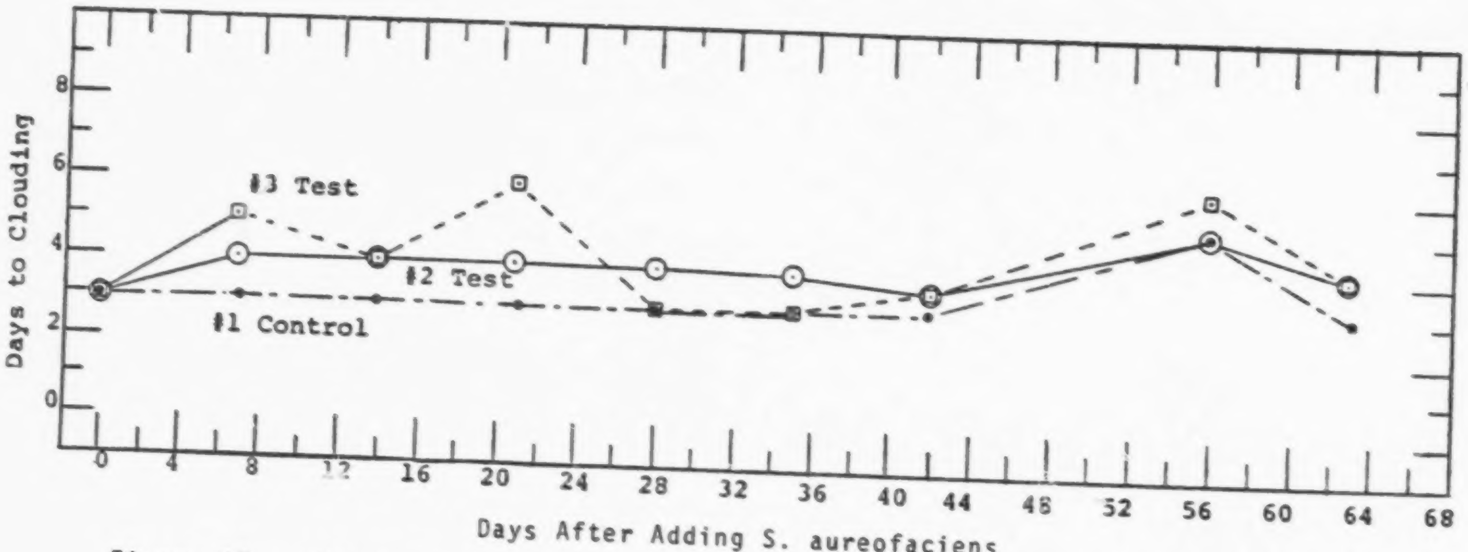


Figure 33 - Effect of S. aureofaciens on Oxidation Times of Robena Bacteria

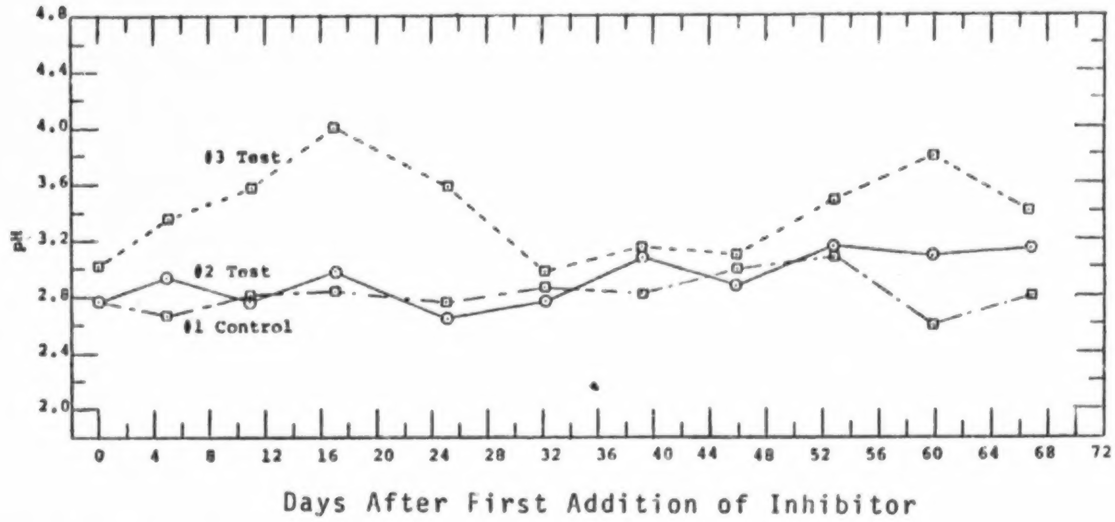


Figure 34 - Effect of S. aureofaciens on pH of Robena Cultures in Upward-flowing Vertical Streams

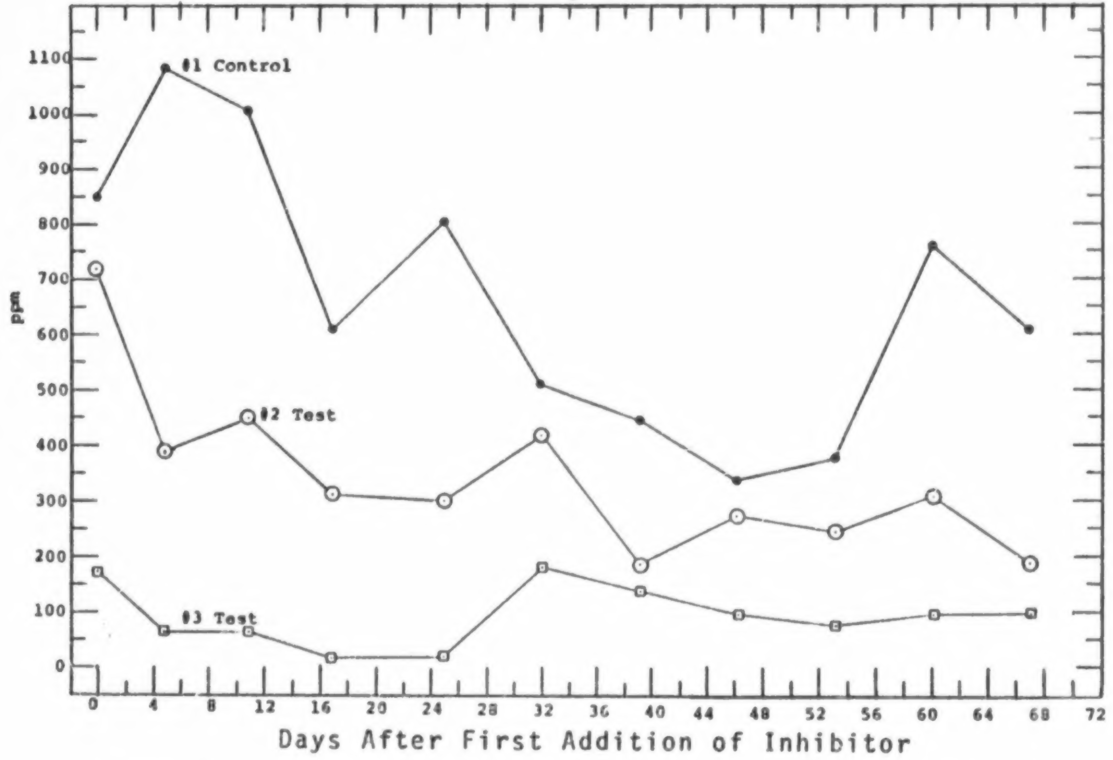


Figure 35 - Effect of S. aureofaciens on Acid Production by Robena Bacteria on Upward-flowing Vertical Streams

SECTION 14

ACKNOWLEDGEMENTS

Dr. Robert P. Zimmerer performed services of electron microscopy and consultant services on overall conduct of the project. Dr. Vincent Gerenscer was consulted on the characteristics of Caulobacters and he furnished four stains for evaluation. This project was greatly added by support of Dr. David R. Maneval, Director of Research and Development for the Pennsylvania Department of Mines and Mineral Industries; of Dr. H. B. Charmbury, Secretary of this department; of Mr. Ronald D. Hill, Chief of Mine Drainage Pollution Control Activities of the Federal Water Pollution Control Administration.

The contributions of the United States Steel Corporation was of great value to the project, and are gratefully acknowledged.

BLANK PAGE

SECTION 15

REFERENCES

Adams, Mark H., Bacteriophages, Interscience Publishers, Inc., New York, 1959

Alexander, M., Introduction to Soil Microbiology, John Wiley and Sons, Inc., 1967

American Public Health Association, Standard Methods for the Examination of Water and Wastewater., 12th Edition, 1965

Bradley, D.E., "Ultrastructure of Bacteriophages and Bacteriocins", Bacteriological Reveiws 33: 230-314, 1967

Bradley, D.E., "The Isolation and Morphology of Some New Bacteriophages Specific for Bacillus and Acetobacter Species", J. Gen. Microbiology 41: 233-241, 1965

Brownlee, K.A., Industrial Experimentation, Chemical Publishing Company, New York,1953

Brownlee, K.A., Statistical Theory and Methodology in Science and Engineering, John Wiley and Sons, New York 1965

Chruchill, Arthur V. and Leathen, William W., "Development of Microbiological Sludge Inhibitors", ASO Technical Report 61-193 September 1962

Gerencser, V.,Personal Communication, May, 1969.

Hach Chemical Company; Ames, Iowa

Hesseltire, C.W., Benedict, R.G., and Pridham, T.G., "Useful Criteria for Species Differentiation in the Genus Streptomyces", Annals of the New York Academy of Sciences, 60, Art. 1 pp 136-151, 1954.

Poindexter, J.S., "Biological Properties and Classification of the Caulobacter Group", Bacteriological Reveiws 28: 231-295, 1964

Shearer, R.E. and Everson, W.A., Elimination of Pollution in Mine Drainage, P.R. No. 2 (Jan-June) Project No. CR-54, Commonwealth of Pennsylvania, Department of Mines and Mineral Industries,

Shearer, R.E. and Everson, W.A., Elimination of Pollution in Mine Drainage, R.R. No. 6 (Jan- June) Project No. CR-62, Commonwealth of Pennsylvania, Department of Mines and Mineral Industries, 1967

REFERENCES (CONTINUED)

Shearer, R.E. et al Second Symposium on Acid Mine Drainage, Coal Industry Advisory Committee, 1968

Stanier, R.Y., Doudoroff, M., Adelberg, E.A., The Microbiological World, Prentice-Hall, Inc., New York 1961

The American Type Culture Collection, Catalogue of Strains. Eighth Edition, 1968.

Waksman, S.A., The Actinomycetes, Ronald Press Company, New York, 1967.

Zimmerer, R.P. - Personal Communication, 1969

SECTION 16

GLOSSARY

Adaption - Any change in an organism which increases its fitness to the environment.

Autocatalytic Inhibitor - An inhibitor produced in the death phase of microorganisms which when applied to fresh viable microorganisms causes them to produce more of the inhibitor and induces death.

Autotrophic - Needing only inorganic compounds for nutrition.

Bacterial Reduction - A decrease in bacterial numbers.

Bactericide - An agent which kills bacteria.

Bacteriocin - A natural class of highly specific antibiotics.

Bacteriophage - Antonomous microbes analogous to plant and animal viruses but obligately parasitic on bacteria.

Bacteriostatic - Prevention of the growth of bacteria without destruction.

Caulobacters - Gram-negative, unicellular, stalked bacteria which multiply by binary fission. During reproduction both stalked and non-stalked forms exist.

Flagella - Whip-like appendages used by bacteria singly or in number for motility.

Filtrate Inhibitor - Inhibitor obtained by inoculating unconcentrated natural inhibitor into a nutrient medium, along with iron bacteria. After incubating, the material is millipore-filtered to isolate the filtrate.

Inhibitor - An agent which slows or interferes with growth of bacteria.

Lysis - Dissolution of the living cells of bacteria.

Mutation - A sudden variation (change) in microorganisms. A transmissible variation that is likely to be permanent, seemingly arising suddenly and spontaneously.

Oxidation Times - The time (in days) required for bacteria to fully oxidize ferrous iron (as part of synthetic mine drainage). This gives some measure of bacterial inhibition as test quantities of iron bacteria and inhibitor are always compared with a control tube of ferrous iron mine drainage containing the same quantity of iron bacteria as the test. A negative control tube is also used with each set of tests in which the ferrous iron should remain unoxidized for long periods of time.

Plaques - Zones of lysis.

Potential Phage Sources - Natural environmental materials such as soil, water, refuse, etc. which might yield quantities of phage upon treatment with the proper bacteria.

Satellite Bacteria - A few sparsely located resistant bacteria appearing in an otherwise clear zone of an antibiotic disk where sensitive bacteria have been lysed.

Seed Culture - A fairly large culture of bacteria growing in a nutrient and aliquots of which are used to inoculate other cultures or systems.

Streptomyces - Members of a genus of the order Actinomycetales. Members of this genus produce most of the antibiotics and are most often soil microorganisms.

Variant - Similar to mutant.

Viable - Able to reproduce themselves.

Viability Count - Numbers of living cells per milliliter of liquid.

Viruses - Infectious filterable particles (usually less than 300 millimicron in diameter) that can reproduce only within a specific host cell.

APPENDIX 1

Statistical Analysis of Inhibition in Various Streams

In order to assess the confidence that inhibition of iron/sulfur bacteria had been obtained in different experiments, a statistical program was set up on a General Electric #400 Time Sharing Programming System. The arithmetic mean and standard deviation of various sets of data were obtained from statistical tables. The results together with the underlying data are given in Table 31.

The data selected for the calculations arbitrarily on what appeared to show possible differences. They covered effluents from upward-flowing streams through flooded coal piles inhibited by natural waters and by cultures of Caulobacters and of Streptomyces.

Of 19 sets of data evaluated, only two fell below the 95% confidence level considered necessary for reasonable assurance that there are true differences and that inhibition was attained. One of these sets was for effect on pH of raw natural inhibitor on acid-producing bacteria from Karen mine effluents. To counteract this questionable level (90%), the effect on both sulfate and total iron production in the same test had a 99.5% confidence level. The other set of data falling short of good confidence was that on effect of one of two tests of S. aureofaciens on pH. The comparison set showed a 99.5% confidence level , and the set on the effect of S. aureofaciens on acid concentration showed a confidence level of 99.5%.

Considerable variations are noted in the time for onset of inhibition and in duration of inhibition. These variations on time are possibly due to variations in resistance of various strains of acid-producing bacteria. Decline in acidity and iron content of the control streams are frequently noted, and this is possibly due to depletion of nutrient pyrite in the coal. Because of limited previous experience, it was not possible to predict in advance when additional inhibition had to be added. This may account for limited periods of inhibition shown in the data.

Table 31 - Statistical Data on Characteristics of Inhibited Streams

Effluent Number	Type of Inhibitor	Stream Characteristic Tested	Value before Inhibition	Interval Tested (Days after Adding Inhibitor)	Number of Values	Mean Value	Standard Deviation	Student's "T" Value	Confidence Level on Difference from Control, %
1	Control for 2,3	ppm acid	90	69-231	25	908.8	858.3	---	---
2	Raw Natural	"	100	"	25	322.08	320.44	3.20	>99.5
3	Raw Filtrate	"	75	"	25	80	74.3	4.81	>99.5
1	Control for 2,3	ppm SO_4	1000	6-188	28	852.5	526.8	---	---
2	Raw Natural	"	975	"	27	481	349	3.07	>99.5
3	Raw Filtrate	"	800	"	27	311.1	157	5.12	>99.5
4	Control for 5,6	pH	3.6	35-169	23	4.0	1.22	---	---
5	Raw Natural	"	4.1	"	23	5.0	1.17	2.84	>99.5
6	Raw Filtrate	"	4.2	"	23	4.8	1.11	2.33	>97.5
1	Control for 2,3	Total iron	7.8	76-216	21	252.5	263	---	---
2	Raw Natural	"	32	"	21	51.3	73.9	3.37	>99.5
3	Raw Filtrate	"	19	"	21	12.3	17.7	4.17	>99.5
4	Control for 5,6	Total iron	7	56-189	20	165.9	253.6	---	---
5	Raw Natural	"	14	"	21	21.31	27.9	2.60	>99
6	Raw Filtrate	"	24	"	21	54	96.4	1.89	>95
8	Caulobacter	Total iron	579	21-114	14	26.36	33.89	6.27	>99.5
9	Control for 8	"	517	"	14	160.9	72.8	---	---
4	Control for 5,6	ppm acid	160	84-189	16	653.4	627.1	---	---
5	Raw Natural	"	106	"	16	143.7	175.3	3.12	>99.5
6	Raw Filtrate	"	120	"	16	220.6	301.2	2.49	>99
10	Control for 11	pH	2.3	41-160	17	3.69	.565	---	---
11	Caulobacter	"	2.45	"	17	6.0	.916	8.85	>99.5
10	Control for 11	ppm acid	5800	41-160	18	208.1	111	---	---
11	Caulobacter	"	4360	"	18	18.4	40.1	6.82	>99.5
1a	Control for 2a, 3a	pH	2.79	5-81	12	2.85	.214	---	---
2a	S. aureofaciens	"	2.79	"	12	2.94	.156	1.18	>80, <90
3a	S. aureofaciens	"	3.05	"	12	3.46	.294	5.81	>99.5
1a	Control for 2a, 3a	ppm acid	850	5-81	12	696.7	253.5	---	---
2a	S. aureofaciens	"	730	"	12	324.2	96.05	4.78	>99.5

APPENDIX 2

Analytical Data on Horizontal Flooded and Vertical Spray Systems

Tables 32 through 42 contain detailed analytical data on attempted inhibition in streams through flooded horizontal systems and downward through vertical unflooded spray systems with natural inhibitor. In column headings with numbering such as 1-1, 1-2, 1-3, etc. the first digit represents the system number. In horizontal systems, the second digit represents the sample port starting at the influent end. Thus 2-4 represents the fourth sample port in system number two. In the vertical systems, the second digit represents the sample port number starting at the top. Thus, 2-4 again represents the fourth port in system number two.

For purposes of economy, not all ports were sampled every time and data are absent as represented by dashes. Some data are also absent because of inability to obtain samples because of some mechanical difficulty.

No evidence of inhibition was seen in the data. Some anomalous values appearing in the tables are due to alkalinity of pyrite added as nutrient to replace depleted pyrite.

Date and Days After Adding Inhibitor		Robena bacteria natural inhibitor									Robena bacteria								
		1-1	1-2	1-3	1-4	1-5	1-6	1-7	1-8	1-9	2-1	2-2	2-3	2-4	2-5	2-6	2-7	2-8	2-9
11/11	-1	220	---	---	---	900	---	---	---	110	360	---	---	---	2200	---	---	---	160
11/21	9	75	---	---	---	120	---	---	---	115	3055	---	---	---	490	---	---	---	570
11/26	14	35	---	---	---	925	---	---	---	140	245	---	---	---	965	---	---	---	1045
12/9	27	65	65	385	780	1040	---	---	---	370	-5	15	70	195	485	---	---	---	785
12/15	34	150	340	865	1355	1974	---	---	---	1750	-20	85	210	235	580	---	---	---	1415
12/26	44	110	35	70	170	240	---	---	---	715	-10	70	120	140	405	---	---	---	1335
1/8	57	250	985	---	2325	3175	---	---	---	2910	-15	120	---	290	605	---	---	---	1630
1/14	63	185	210	---	1615	2855	---	---	---	4525	-5	105	---	205	505	---	---	---	1635
1/21	70	140	420	---	1485	2460	---	---	---	5425	25	60	---	145	285	---	---	---	860
1/28	77	135	445	---	1365	2270	--	---	---	6230	10	70	---	180	300	---	---	---	1035
2/5	85	35	105	---	320	565	---	---	---	5405	25	55	---	145	320	---	---	---	890
2/11	91	20	40	---	135	275	---	---	---	4580	20	35	---	45	120	---	---	---	420
2/18	98	70	205	---	475	415	---	---	---	940	25	125	---	15	165	---	---	---	565
2/26	106	15	35	---	360	605	---	---	---	132	20	60	---	10	285	---	---	---	815
3/5	0	30	100	---	240	540	---	---	---	1190	20	50	---	130	250	---	---	---	720
3/11	6	45	235	---	545	1285	---	---	---	1240	100	65	---	50	320	---	---	---	905
3/18	13	20	115	---	245	575	---	---	---	910	30	105	---	320	545	---	---	---	1385
3/25	20	40	190	---	340	695	---	---	---	1045	40	310	---	430	625	---	---	---	1695
4/1	27	50	120	---	205	515	---	---	---	780	20	155	---	390	605	---	---	---	1530
4/8	34	30	260	---	185	430	---	---	---	630	25	105	---	345	550	---	---	---	1615
4/15	41	40	110	---	125	340	---	---	---	420	20	250	---	110	490	---	---	---	1050
4/22	48	50	160	---	285	590	---	---	---	885	25	335	---	380	420	---	---	---	1040
4/29	55	60	260	---	520	815	---	---	---	1145	45	255	---	255	485	---	---	---	1230
5/6	62	30	180	---	130	330	---	---	---	460	35	215	---	160	365	---	---	---	720
5/14	70	100	115	---	105	255	---	---	---	325	30	230	---	80	300	---	---	---	755
5/20	76	46	130	---	140	310	---	---	---	400	14	410	---	235	300	---	---	---	1010
5/26	82	26	102	---	94	265	---	---	---	360	4	295	---	305	435	---	---	---	855
6/3	90	19	171	---	178	36	---	---	---	550	12	150	---	230	420	---	---	---	700
6/12	85	68	18	---	8	11	---	---	---	385	10	7	---	13	14	---	---	---	450

Table 32 - Total Acidity (ppm $CaCO_3$ equiv.)
Along Horizontal System

Date and Days After Adding Inhibitor		Robena bacterial natural inhibitor									Robena bacteria								
		1-1	1-2	1-3	1-4	1-5	1-6	1-7	1-8	1-9	2-1	2-2	2-3	2-4	2-5	2-6	2-7	2-8	2-9
11/11	-1	45	---	---	---	200	---	---	---	25	125	---	---	---	470	---	---	---	5
11/21	9	10.4	---	---	---	36.4	---	---	---	17.7	192	---	---	---	177	---	---	---	132
11/26	14	2.6	---	---	---	117	---	---	---	1.0	50	---	---	---	250	---	---	---	145
12/3	21	94	350	395	598	760	---	---	---	43	30	350	335	343	590	---	---	---	398
12/9	27	18	44	48	79	105	---	---	---	98	1	17	11	9	41	---	---	---	210
12/16	34	38	29	141	290	276	---	---	---	228	0	3.6	34	50	79	---	---	---	279
12/26	44	16	29	155	325	580	---	---	---	600	0.3	6.2	12.0	8.8	71	---	---	---	490
1/8	57	95	224	---	650	605	---	---	---	715	0	23	59	---	142	---	---	---	525
1/14	63	15	29	---	390	815	---	---	---	940	0.2	23	---	54	114	---	---	---	433
1/21	70	12.5	63	---	327	518	---	---	---	618	0.1	5.2	---	12	38.5	---	---	---	150
1/28	77	5.7	65	---	295	460	---	---	---	658	0	4.4	---	27	47	---	---	---	204
2/5	85	23	6	---	48	83	---	---	---	645	4.7	6.8	---	20	45	---	---	---	158
2/11	91	0	0	---	11	31	---	---	---	630	0	0	---	1	11	---	---	---	55
2/18	98	0.1	3.1	---	1.8	0.1	---	---	---	45	0	0.3	---	0.1	0.1	---	---	---	0.4
2/26	106	0.3	0.3	---	66	110	---	---	---	254	3.1	4.4	---	56	76	---	---	---	269
3/5	0	0	0	---	36	93	---	---	---	214	3.6	1	---	8.3	41	---	---	---	18
3/11	6	2.6	4.2	---	48	160	---	---	---	300	7.3	6.2	---	28	31	---	---	---	183
3/18	13	0.3	11	---	36	88	---	---	---	179	5.2	12.2	---	47	97	---	---	---	271
3/25	20	1.6	10	---	43	107	---	---	---	182	0	39	---	60	144	---	---	---	337
4/1	27	1.6	8	---	24	71	---	---	---	139	1.8	16	---	61	104	---	---	---	305
4/8	34	0	33	---	22	53	---	---	---	103	0.1	10	---	53	91	---	---	---	353
4/15	41	1.1	8	---	14	47	---	---	---	67	0.2	36	---	24	90	---	---	---	228
4/22	48	3.4	18	---	41	113	---	---	---	150	2	56	---	67	68	---	---	---	240
4/29	55	12	47	---	117	63	---	---	---	307	2	42	---	48	97	---	---	---	323
5/6	62	3	33	---	11	53	---	---	---	83	26	31	---	26	64	---	---	---	153
5/14	70	16	14	---	8	35	---	---	---	50	1	40	---	9	57	---	---	---	164
5/20	76	4	10	---	13	50	---	---	---	62	2	84	---	40	53	---	---	---	257
5/26	82	1	12	---	8	42	---	---	---	66	0.5	55	---	63	70	---	---	---	190
6/3	90	4	30	---	10	57	---	---	---	114	0	21	---	37	83	---	---	---	73
6/12	99	0.5	2	---	6	35	---	---	---	64	0	2	---	7	14	---	---	---	78

Table 33 - Total Iron Along Horizontal System (ppm)

Date and Days After Adding Inhibitor		Robena bacteria natural inhibitor									Robena bacteria								
		1-1	1-2	1-3	1-4	1-5	1-6	1-7	1-8	1-9	2-1	2-2	2-3	2-4	2-5	2-6	2-7	2-8	2-9
11/11	-1	30	---	---	---	62	---	---	---	8	100	---	---	192		---	---	---	10
11/21	9	0	---	---	---	3	---	---	---	0	33	---	---	---	64	---	---	---	38
11/26	14	0	---	---	---	43	---	---	---	0	38	---	---	---	148	---	---	---	35
12/3	21	44	148	63	67	205	---	---	---	15	9.5	153	280	285	230	---	---	---	78
12/9	27	0	0	3	8	48	---	---	---	40	0	0	0	14	29	---	---	---	27
12/16	34	0.5	16	10	15	81	---	---	---	26	0	0	6.5	12	68	---	---	---	59
12/26	44	0	0	7.8	3.3	20.3	---	---	---	46.5	0	0	0	0	9.5	---	---	---	12.5
1/8	57	20	109	---	115	167	---	---	---	326	0	53	---	48	82	---	---	---	60
1/14	63	0	0	---	3.5	11	---	---	---	42	0	9.5	---	38.5	27.5	---	---	---	48.5
1/21	70	0.5	29	---	12	43	---	---	---	222	0	0.3	---	8.0	16.3	---	---	---	27.5
1/28	77	0	19	---	5.5	29	---	---	---	143	0	1.0	---	16	10	---	---	---	27
2/5	85	0	0	---	2	8.5	---	---	---	98	0	2	---	13	18	---	---	---	25
2/11	91	0	0	---	0	0	---	---	---	70	0	0	---	0.1	0.1	---	---	---	4
2/18	98	0	1.0	---	1.8	0	---	---	---	3.3	0	0	---	0.1	0	---	---	---	0
2/26	106	0	0	---	43	41	---	---	---	46	0	0	---	47.3	68	---	---	---	222
3/5	0	0	0	---	1	23	---	---	---	35	0	0	---	0	5	---	---	---	10
3/11	6	0	0	---	12	30	---	---	---	73	0.1	0.1	---	17	7.5	---	---	---	12
3/18	13	0	0	---	6.5	10.5	---	---	---	24	0	0.1	---	15	14	---	---	---	41
3/25	20	0	0	---	13	15	---	---	---	40	0	2.5	---	33	2.8	---	---	---	101
4/1	27	0.1	0.1	---	12	20	---	---	---	38	0	8	---	50	28	---	---	---	52
4/8	34	0	0	---	2.5	1.8	---	---	---	54	0	1.8	---	40	7	---	---	---	70
4/15	41	0	0	---	1.1	1.8	---	---	---	20	0	1.1	---	15	3.8	---	---	---	47
4/22	48	0	0	---	12	15	---	---	---	20	0	1	---	30	7	---	---	---	31
4/29	55	0.1	0.5	---	70	45	---	---	---	81	0	5	---	29	23	---	---	---	53
5/6	62	0	0	---	0	0.1	---	---	---	0.5	0	0	---	0.2	0.2	---	---	---	6
5/14	70	4	0	---	0.5	6	---	---	---	5	0	3	---	3	16	---	---	---	28
5/20	76	0	0	---	0	0.5	---	---	---	2	0	8	---	29	6	---	---	---	65
5/26	82	0	0	---	0	6	---	---	---	5	0	6	---	46	22	---	---	---	32
6/3	90	0	8	---	4	12	---	---	---	11	0	15	---	25	10	---	---	---	16
6/12	99	0	0	---	2	9	---	---	---	11	0	0	---	5	10	---	---	---	15

Table 34 - Ferrous Iron Along Horizontal System (ppm)

Date and Days After Adding Inhibitor		Robena bacteria natural inhibitor									Robena bacteria								
		1-1	1-2	1-3	1-4	1-5	1-6	1-7	1-8	1-9	2-1	2-2	2-3	2-4	2-5	2-6	2-7	2-8	2-9
11/12	0	3	---	---	---	3	---	---	---	4	3	---	---	---	3	---	---	---	4
11/14	2	4	---	---	---	4	---	---	---	4	4	---	---	---	4	---	---	---	4
11/21	9	4	---	---	---	5	---	---	---	6	6	---	---	---	4	---	---	---	5
11/27	15	4-5	---	---	---	4-5	---	---	---	4-5	4-5	---	---	---	4-5	---	---	---	4-5
12/3	21	3	3	3	3	3	---	---	---	4	---	3	3	3	3	---	---	---	3
12/10	28	3	3	3	3	3	---	---	---	3	3	3	3	3	3	---	---	---	3
12/18	36	4-5	4-5	4-5	4-5	2	---	---	---	2	4-5	4-5	4-5	4-5	4-5	---	---	---	4-5
1/2/69	51	3-4	3-4	3-4	5	3-4	---	---	---	3-4	---	---	---	---	3-4	---	---	---	3-4
1/9	58	3-4	3-4	3-4	3-4	3-4	---	---	---	3-4	6	3-4	5	3-4	3-4	---	---	---	3-4
1/16	65	4	4	4	4	4	---	---	---	4	6	4	5	5	4	---	---	---	4
1/23	72	4	4	4	4	4	---	---	---	4	6	6	6	6	6	---	---	---	6
1/30	79	4	4	4	4	4	---	---	---	4	6	4	5	4	4	---	---	---	4
2/6	87	4	4	4	4	4	---	---	---	4	4	6	6	4	4	---	---	---	4
2/13	94	6	6	11	6	5	---	---	---	4	8	5	6	6	6	---	---	---	4
2/20	101	4	4	5	4	4	---	---	---	4	5	4	5	7	4	---	---	---	5
2/27	108	4	4	4	4	4	---	---	---	4	4	4	5	4	4	---	---	---	4
3/5	114	4	4	4	4	4	---	---	---	4	4	4	4	4	4	---	---	---	4
3/14	123	3	3	3	3	3	---	---	---	3	3	3	3	3	3	---	---	---	3
3/20	129	3	3	3	3	3	---	---	---	3	3	3	3	3	3	---	---	---	3
3/27	136	3-4	3-4	3-4	3-4	5	---	---	---	5	3-4	3-4	5	3-4	3-4	---	---	---	4
4/3	143	3	3	3	3	5	---	---	---	5	5	3	4	4	3	---	---	---	3
4/11	151	3	4	3	4	5	---	---	---	5	5	4	5	5	4	---	---	---	4
4/17	157	3-4	3-4	3-4	3-4	3-4	---	---	---	4	4	3-4	5	3-4	3-4	---	---	---	3-4
4/25	165	3	3	3	4	4	---	---	---	4	4	4	4	4	3	---	---	---	4
5/1	171	3	3	3	3	5	---	---	---	5	3	3	4	3	3	---	---	---	3
5/8	178	3-4	3-4	3-4	3-4	4	---	---	---	4	4	3-4	3-4	4	4	---	---	---	5
5/15	185	3-4	3-4	3-4	3-4	3-4	---	---	---	5	3-4	3-4	3-4	3-4	3-4	---	---	---	3-4
5/22	192	3-4	3-4	3-4	3-4	5	---	---	---	5	3-4	3-4	5	5	3-4	---	---	---	5
5/29	199	3-4	3-4	3-4	3-4	3-4	---	---	---	3-4	3-4	3-4	3-4	3-4	3-4	---	---	---	3-4
6/6	207	3	3	3	3	3	---	---	---	3	3	3	3	3	3	---	---	---	3
6/12	213	3	3	3	3	5	---	---	---	5	5	3	4	3	3	---	---	---	5
6/19	220	5	3	3	3	3	---	---	---	3	5	3	3	3	5	---	---	---	5
6/26	227	3	3	3	3	3	---	---	---	3	3	3	3	3	3	---	---	---	3

Table 35 - Oxidation Times of Bacteria From Along Horizontal System (days)

Date and Days After Adding Inhibitor		Robena bacteria natural inhibitor									Robena bacteria									Tap
		1-1	1-2	1-3	1-4	1-5	1-6	1-7	1-8	1-9	2-1	2-2	2-3	2-4	2-5	2-6	2-7	2-8	2-9	H_2O
11/11	-1	3.0	3.2	3.1	3.1	2.9	3.0	3.0	3.6	6.7	3.0	3.6	2.0	2.0	2.6	3.0	3.0	3.3	5.4	
11/15	3	3.0	3.1	3.1	3.1	3.0	3.0	3.1	3.4	4.2	6.2	3.4	2.4	2.8	2.7	2.7	3.0	3.1	3.3	
11/19	7	3.0	3.0	2.8	3.1	3.0	3.0	3.0	3.1	3.8	6.5	3.1	3.0	3.2	2.7	2.7	2.9	3.0	3.0	
11/25	13	4.8	3.4	3.3	3.2	3.2	3.0	3.3	3.2	3.4	2.2	2.6	3.0	3.2	2.8	2.8	2.9	3.0	3.0	
11/26	14	3.8	3.6	3.4	3.2	2.9	3.0	3.2	3.2	3.5	3.2	2.8	3.2	3.4	3.0	3.0	3.1	3.1	3.1	
12/4	22	3.0	2.7	2.8	2.6	2.6	2.9	2.9	2.9	3.1	3.7	2.7	3.0	3.0	2.7	2.8	2.8	2.8	2.8	
12/9	27	2.9	3.3	3.2	3.2	3.0	3.9	3.3	2.9	3.1	6.3	3.4	3.6	3.6	3.2	3.1	3.2	2.8	2.8	
12/12	30	3.4	3.4	3.0	2.8	2.7	2.8	3.0	2.8	3.0	6.3	3.8	3.5	3.2	2.9	2.9	3.0	2.8	2.9	7.1
12/16	34	3.0	3.0	2.9	2.7	2.6	2.8	2.9	2.9	2.8	6.3	3.5	3.3	3.2	3.0	3.0	3.0	2.8	2.9	7.1
1/2	51	3.2	3.2	3.1	3.0	2.9	3.1	3.3	3.0	3.1	6.6	3.6	3.6	3.5	3.2	3.2	3.1	3.0	3.0	7.1
1/13	62	3.1	2.9	2.8	2.7	2.6	2.7	2.9	2.7	2.7	6.2	3.5	3.3	3.2	3.0	2.9	2.9	2.9	2.9	7.0
1/20	69	3.2	3.0	2.9	2.7	2.6	2.6	2.7	2.7	2.6	6.1	3.7	3.6	3.6	3.1	3.1	3.0	2.8	2.8	7.0
1/27	76	3.2	3.0	2.9	2.8	2.6	2.6	2.7	2.6	2.6	4.5	3.7	3.6	3.6	3.2	3.1	3.0	2.9	2.9	7.0
2/5	85	3.2	2.9	2.4	2.5	2.3	---	---	---	2.4	3.1	2.8	2.9	2.5	2.2	---	---	---	2.1	
2/11	91	3.95	3.5	---	2.9	2.65	---	---	---	2.25	3.95	3.55	---	3.4	2.95	---	---	---	2.6	
2/12	92	3.9	3.6	3.4	3.4	3.2	3.1	3.3	3.0	2.8	5.4	4.1	3.9	3.9	3.6	3.4	3.5	3.1	3.2	7.1
2/20	100	6.3	3.7	6.8	3.1	3.0	6.8	6.8	7.0	2.9	4.9	3.4	6.9	6.2	3.6	6.6	6.3	7.0	6.3	
2/26	106	6.2	3.7	---	2.8	2.6	---	---	---	2.4	4.0	4.2	---	5.6	3.3	---	---	---	2.6	
3/5	0	5.8	3.6	---	2.8	2.7	---	---	---	2.7	4.2	3.6	---	3.0	2.9	---	---	---	2.5	
3/12	7	4.0	3.8	---	2.8	2.8	---	---	---	2.7	3.5	3.5	---	3.2	3.2	---	---	---	2.8	
3/18	13	4.8	3.65	---	3.4	3.1	---	---	---	2.95	4.45	3.72	---	3.35	3.2	---	---	---	2.87	
3/25	20	3.5	2.9	---	265	245	---	---	---	2.3	3.5	2.65	---	2.5	2.42	---	---	---	2.15	
4/1	27	3.25	2.95	---	2.8	2.6	---	---	---	2.45	3.7	2.9	---	2.6	2.5	---	---	---	2.3	
4/8	34	3.35	2.95	---	2.95	2.75	---	---	---	2.6	3.72	3.1	---	2.8	2.65	---	---	---	2.4	
4/15	41	3.4	3.0	---	3.0	2.62	---	---	---	2.55	4.35	2.75	---	2.9	2.6	---	---	---	2.3	
4/22	48	3.25	2.9	---	2.7	2.5	---	---	---	2.4	3.7	2.7	---	2.6	2.55	---	---	---	2.3	
4/29	55	3.2	2.72	---	2.45	2.3	---	---	---	2.22	3.5	2.7	---	2.7	2.5	---	---	---	2.2	
5/6	62	3.4	2.9	---	2.9	2.6	---	---	---	2.5	3.7	2.7	---	2.9	2.6	---	---	---	2.4	
5/14	70	3.12	3.0	---	2.95	2.65	---	---	---	2.6	3.95	2.8	---	3.15	2.75	---	---	---	2.35	
5/20	76	3.45	3.0	---	2.95	2.6	---	---	---	2.6	4.95	2.6	---	2.75	2.6	---	---	---	2.25	
5/26	82	3.65	3.12	---	3.0	2.7	---	---	---	2.6	5.55	2.7	---	2.65	2.6	---	---	---	2.4	
6/3	90	3.9	3.1	---	2.9	2.6	---	---	---	2.45	5.45	2.95	---	2.8	2.6	---	---	---	2.45	
6/12	99	6.8	6.9	---	6.7	6.55	---	---	---	2.55	6.7	6.9	---	6.75	6.65	---	---	---	2.5	

Table 36 - pH Along Horizontal System

Date and Days After Adding Inhibitor		Robena bacteria natural inhibitor									Robena bacteria								
		1-1	1-2	1-3	1-4	1-5	1-6	1-7	1-8	1-9	2-1	2-2	2-3	2-4	2-5	2-6	2-7	2-8	2-9
11/11	-1	500	---	---	---	2225	---	---	---	1860	1400	---	---	---	2750	---	---	---	1700
11/21	9	400	---	---	---	825	---	---	---	1425	3250	---	---	---	1425	---	---	---	1125
11/26	14	325	---	---	---	850	---	---	---	1175	700	---	---	---	1500	---	---	---	1800
12/3	21	675	1675	1950	3125	3675	---	---	---	2150	1250	1775	1975	1750	1525	---	---	---	2800
12/9	27	275	100	350	425	650	---	---	---	1375	1250	150	150	275	475	---	---	---	1700
12/16	34	300	300	650	950	1400	---	---	---	1675	100	150	300	400	600	---	---	---	2175
12/26	44	100	275	600	950	1575	---	---	---	2200	110	85	75	200	400	---	---	---	2000
1/8	57	550	800	---	1975	1825	---	---	---	2450	125	125	---	275	550	---	---	---	2525
1/14	63	125	110	---	115	2175	---	---	---	5050	135	135	---	200	550	---	---	---	2175
1/21	70	150	275	---	875	1450	---	---	---	3625	125	115	---	100	260	---	---	---	475
1/28	77	100	450	---	950	1550	---	---	---	4150	50	50	---	250	275	---	---	---	825
2/5	85	100	100	---	275	475	---	---	---	3250	125	150	---	140	200	---	---	---	550
2/11	91	140	150	---	275	250	---	---	---	2400	125	275	---	150	225	---	---	---	425
2/18	98	100	275	---	950	875	---	---	---	1125	250	950	---	750	750	---	---	---	850
2/26	106	200	200	---	400	575	---	---	---	1050	150	125	---	300	400	---	---	---	850
3/5	0	150	150	---	300	450	---	---	---	1000	100	100	---	200	350	---	---	---	1000
3/11	6	150	75	---	250	600	---	---	---	925	125	50	---	140	275	---	---	---	650
3/18	13	50	25	---	25	425	---	---	---	750	1000	125	---	160	375	---	---	---	850
3/25	20	100	125	---	175	475	---	---	---	750	125	250	---	425	475	---	---	---	1025
4/1	27	150	200	---	140	450	---	---	---	600	150	175	---	400	600	---	---	---	1075
4/8	34	100	500	---	250	350	---	---	---	500	125	100	---	425	550	---	---	---	1000
4/15	41	125	60	---	135	300	---	---	---	275	145	350	---	150	400	---	---	---	850
4/22	48	125	100	---	300	550	---	---	---	575	125	275	---	425	500	---	---	---	850
4/29	55	50	175	---	700	825	---	---	---	1000	150	300	---	150	100	---	---	---	1125
5/6	62	150	135	---	125	250	---	---	---	250	60	30	---	90	175	---	---	---	600
5/14	70	50	125	---	125	225	---	---	---	175	175	175	---	50	40	---	---	---	625
5/20	76	125	125	---	150	275	---	---	---	400	125	425	---	265	300	---	---	---	850
5/26	82	125	100	---	100	150	---	---	---	125	125	275	---	125	125	---	---	---	710
6/3	90	125	250	---	50	295	---	---	---	500	120	100	---	250	300	---	---	---	410
6/12	99	950	1000	---	850	375	---	---	---	500	1000	600	---	875	750	---	---	---	575

Table 37 - Sulfates Along Horizontal System (ppm)

Date and Days After Adding Inhibitor to 2,4 & 5		Ferrobacillus sulfooxidans no innibitor							Ferrobacillus sulfooxidans natural inhibitor						
		1-1	1-2	1-3	1-4	1-5	1-6	1-7	2-1	2-2	2-3	2-4	2-5	2-6	2-7
11/19	-8	4.7	---	86.3	---	---	65.0	---	10.9	---	34.3	---	24.4	---	---
12/4	7	0.5	---	4.7	---	5.7	---	5.2	4.7	---	1.2	---	13.0	---	14.6
12/9	12	5.7	---	6.2	---	5.2	---	3.6	2.6	---	3.6	---	4.7	---	3.4
12/18	21	2.6	---	7.3	---	47.8	---	17.7	8.3	---	3.1	0.5	---	---	23.1
1/2/69	36	19.0	---	28.6	---	17.7	---	8.3	0.2	---	15.6	1.0	---	---	11.2
1/9	43	---	0.0	---	0.2	---	26.8	9.9	---	5.2	---	0.0	---	---	13.8
1/16	50	---	1.0	2.1	---	---	25.0	56.0	---	0.2	---	0.0	---	---	27.0
1/23	57	0.0	0.3	---	---	125.0	---	48.0	0.3	0.0	---	4.0	---	---	26.0
1/31	65	0.0	0.2	20.3	---	---	108.0	63.0	0.0	0.0	0.0	2.3	---	---	42.6
2/6	71	7.8	1.8	13.0	---	114.8	---	56.7	2.3	3.1	5.2	6.5	---	---	44.5
2/14	79	0.0	0.0	0.0	---	---	136.0	68.0	0.0	0.0	2.6	3.6	---	---	52.0
2/20	85	8.3	1.8	33.3	---	---	---	267.0	---	0.0	4.7	6.0	---	---	83.0
2/27	92	0.5	10.9	137.0	---	---	285.0	325.0	0.3	0.5	4.7	7.8	---	---	101.0
3/6	99	0.5	61.4	158.0	545.0	---	---	545.0	0.0	5.2	15.6	7.8	---	---	230.0
3/13	106	0.0	56.0	---	3.6	---	138.0	31.0	0.3	7.5	67.0	30.0	---	---	119.0
3/20	113	0.0	0.0	---	6.8	58.0	---	20.0	5.2	0.3	44.0	24.0	---	---	117.0

		Ferrobacillus ferrooxidans no inhibitor							Ferrobacillus ferrooxidans natural inhibitor						
		3-1	3-2	3-3	3-4	3-5	3-6	3-7	4-1	4-2	4-3	4-4	4-5	4-6	4-7
11/19	-8	---	---	121.0	---	145.0	---	74.0	3.1	---	113.0	---	68.-	---	---
12/4	7	0.0	---	8.0	---	15.0	---	17.0	1.0	---	3.0	---	3.0	---	10.0
12/9	12	---	2.9	---	---	12.2	---	6.2	8.3	---	51.5	---	3.0	---	11.0
12/18	21	10.7	---	---	---	0.0	---	13.0	0.0	---	0.0	---	---	0.5	54.6
1/2/69	36	---	5.5	---	---	8.8	---	21.0	10.0	---	10.0	---	---	79.0	155.0
1/9	43	---	---	---	---	15.6	---	34.3	---	0.0	---	0.5	---	91.0	113.0
1/16	50	---	94.0	---	---	18.0	---	98.0	0.2	---	0.5	---	---	128.0	85.0
1/25	57	---	---	2.0	---	17.0	---	51.0	0.1	0.2	---	0.8	---	---	125.0
1/31	65	---	---	---	36.0	12.0	---	52.0	4.4	0.3	---	0.3	---	73.0	80.0
2/6	71	---	---	---	---	13.5	13.0	46.7	23.4	2.3	5.2	4.2	---	---	78.5
2/14	79	---	---	---	---	15.6	---	62.4	0.0	0.0	0.5	---	---	67.0	107.0
2/20	85	---	---	---	---	85.0	109.0	253.0	0.1	---	7.3	---	---	106.0	210.0
2/27	92	0.5	268.0	---	215.0	75.0	---	34.0	1.6	3.1	---	2.6	---	137.0	76.0
3/6	99	---	---	230.0	---	17.0	---	68.0	0.0	0.0	---	0.1	---	70.0	114.0
3/13	106	1.8	---	---	---	22.9	---	417.0	0.0	0.1	---	5.7	---	94.9	41.0
3/20	113	0.0	---	---	---	18.0	---	587.0	0.0	0.0	---	0.0	---	36.0	43.0

Table 38 - Total Iron (ppm) Through Vertical Spray System

Date and Days After Adding Inhibitor to 2,4 & 5		Mixed species natural inhibitor							Mixed species no inhibitor						
		5-1	5-2	5-3	5-4	5-5	5-6	5-7	6-1	6-2	6-3	6-4	6-5	6-6	6-7
11/19	-8	---	---	---	---	31.0	---	60.0	1.8	---	43.0	---	20.0	---	---
12/4	7	0.0	---	3.0	---	9.0	---	13.0	0.0	---	1.0	---	0.5	---	1.6
12/9	12	---	3.0	---	9.0	---	9.0	13.0	0.0	---	1.0	---	1.0	---	2.0
12/18	21	---	---	23.1	---	11.4	---	7.3	10.4	---	4.4	---	8.8	---	2.8
1/2/69	36	---	225.0	111.0	---	22.4	---	343.0	0.3	---	0.0	---	3.6	---	19.8
1/9	43	---	328.0	---	---	---	114.0	55.0	---	0.2	---	1.0	---	0.0	17.0
1/16	50	---	---	428.0	320.0	---	51.0	80.0	---	0.2	0.3	---	---	---	8.8
1/23	57	0.0	---	600.0	355.0	255.0	---	91.0	0.0	0.0	---	0.2	11.0	---	7.8
1/31	65	---	---	58.0	166.0	75.0	80.0	11.0	0.3	1.8	---	0.0	13.0	---	6.8
2/6	71	---	---	107.0	151.0	61.4	70.2	32.0	2.3	2.3	---	3.1	17.7	---	---
2/14	79	---	---	---	---	173.0	130.0	69.0	0.0	0.0	---	0.1	26.0	---	41.0
2/20	85	---	---	---	535.0	281.0	257.0	133.0	0.3	0.0	0.0	---	28.8	---	26.0
2/27	92	---	395.0	---	343.0	265.0	245.0	116.0	0.1	0.1	---	1.0	26.0	---	35.0
3/6	99	---	---	280.0	450.0	260.0	335.0	275.0	0.0	0.0	---	10.4	32.0	---	94.0
3/13	106	---	---	275.0	465.0	550.0	535.0	20.0	0.0	0.5	---	8.3	---	---	31.0
3/20	113	0.0	---	43.0	136.0	---	151.0	33.0	0.0	0.0	---	0.2	---	42.0	31.0

		No added bacteria						
		7-1	7-2	7-3	7-4	7-5	7-6	7-7
11/19	-8	86.0	---	54.0	---	---	61.0	---
12/4	7	3.0	---	87.0	---	116.0	---	54.0
12/9	12	3.0	---	20.0	---	49.0	---	54.0
12/18	21	3.4	---	49.7	---	47.8	---	38.0
1/2/69	36	0.3	---	2.6	---	37.4	---	14.6
1/9	43	---	0.3	---	4.2	---	15.0	13.0
1/16	50	---	1.0	---	16.6	23.7	---	23.1
1/23	57	0.0	0.0	---	3.1	17.0	---	16.0
1/31	65	0.0	0.0	---	22.0	31.0	---	55.0
2/6	71	3.1	2.3	---	28.6	44.5	---	62.1

Table 38 - Total Iron (ppm) Through Vertical Spray System (Cont.)

Date and Days After Adding Inhibitor to 2,4 & 5		Ferrobacillus sulfooxidans no inhibitor							Ferrobacillus sulfooxidans natural inhibitor						
		1-1	1-2	1-3	1-4	1-5	1-6	1-7	2-1	2-2	2-3	2-4	2-5	2-6	2-7
11/19	-8	---	---	56.0	---	---	48.0	---	---	---	4.5	---	---	17.8	---
12/4	7	0.0	---	0.0	---	0.0	---	0.0	0.0	---	0.0	---	0.0	---	0.0
12/9	12	2.5	---	4.0	---	0.0	---	0.0	0.0	---	1.0	---	0.5	---	0.0
12/18	21	0.0	---	0.0	---	40.0	---	---	0.0	---	0.0	0.0	---	---	---
1/2/69	36	0.2	---	0.0	---	0.0	---	0.0	0.0	---	0.0	0.0	---	---	0.0
1/9	43	0.0	0.0	---	0.0	---	2.5	1.0	---	0.0	---	0.0	---	---	2.0
1/16	50	---	0.0	0.0	---	---	0.6	17.8	---	0.0	---	0.0	---	---	3.5
1/23	57	0.0	0.0	---	---	6.0	---	3.3	0.0	0.0	---	0.2	---	---	1.8
1/31	65	0.0	0.0	1.0	---	---	3.0	14.5	0.0	0.0	0.0	0.0	---	---	13.0
2/6	71	0.0	0.0	0.5	---	7.5	---	12.5	0.0	0.0	0.0	0.0	---	---	9.5
2/14	79	0.0	0.0	0.0	---	---	11.0	22.0	0.0	0.0	0.0	0.0	---	---	15.0
2/21	86	0.1	0.3	0.5	---	---	---	113.0	---	0.0	0.0	0.1	---	---	28.0
2/27	92	0.0	0.0	2.0	---	---	8.0	58.0	0.0	0.0	0.0	0.0	---	---	2.0
3/6	99	0.0	7.0	8.5	13.0	---	---	92.5	0.0	0.0	0.0	0.0	---	---	74.0
3/13	106	0.0	5.5	---	0.0	---	18.0	9.5	0.0	0.8	13.0	7.5	---	---	7.5
3/20	113	0.0	0.0	---	0.0	10.0	---	6.0	0.0	0.0	8.5	3.5	---	---	38.0

		Ferrobacillus ferrooxidans no inhibitor							Ferrobacillus ferrooxidans natural inhibitor						
		3-1	3-2	3-3	3-4	3-5	3-6	3-7	4-1	4-2	4-3	4-4	4-5	4-6	4-7
11/19	-8	---	---	63.0	---	78.0	---	61.0	---	---	7.5	---	---	35.0	---
12/4	7	0.0	---	0.0	---	10.0	---	6.0	0.0	---	0.0	---	0.0	---	2.5
12/9	12	---	0.0	---	---	8.0	---	5.3	0.0	---	5.5	---	0.0	---	2.0
12/18	21	0.0	---	---	---	0.0	---	---	0.0	---	0.0	---	---	0.0	---
1/2/69	36	---	0.0	---	---	0.0	---	0.3	0.0	---	0.0	---	---	3.3	22.5
1/9	43	---	---	---	---	0.3	---	7.5	---	0.0	---	0.2	---	12.5	27.5
1/16	50	---	3.3	---	---	0.2	---	33.8	0.0	---	0.0	---	---	16.8	60.5
1/23	57	---	0.0	---	---	0.5	---	4.5	0.0	0.0	---	0.0	---	---	26.5
1/31	65	0.0	2.3	---	3.5	0.3	---	14.3	0.3	0.3	---	0.3	---	27.0	34.0
2/6	71	---	---	---	---	0.1	0.1	10.5	0.0	0.0	0.0	0.0	---	---	25.5
2/14	79	---	---	---	---	0.1	---	28.0	0.0	0.0	0.0	---	---	14.0	47.5
2/20	85	---	---	---	---	16.5	16.5	72.5	0.0	---	1.0	---	---	14.5	77.0
2/26	92	0.0	5.0	---	6.1	2.3	---	1.8	0.0	0.0	---	0.0	---	5.5	13.0
3/6	99	---	---	10.5	---	0.1	---	20.0	0.0	0.0	---	0.0	---	5.0	33.5
3/13	106	0.0	---	---	---	8.0	---	160.0	0.0	0.0	---	0.5	---	15.5	15.5
3/20	113	0.0	---	---	---	3.5	---	108.0	0.0	0.0	---	0.0	---	8.5	13.0

Table 39 - Ferrous Iron (ppm) Through Vertical Spray System

Date and Days After Adding Inhibitor to 2,4 & 5		Mixed species natural inhibitor							Mixed species no inhibitor						
		5-1	5-2	5-3	5-4	5-5	5-6	5-7	6-1	6-2	6-3	6-4	6-5	6-6	6-7
11/19	-8	---	---	5.0	---	8.0	---	26.0	---	---	7.5	---	---	1.8	---
12/4	7	0.0	---	0.0	---	1.75	---	5.5	0.0	---		---	0.0	---	0.0
12/9	12	---	0.0	---	1.0	---	4.5	5.0	0.0	---	0.0	---	0.0	---	0.0
12/18	21	---	---	0.0	---	0.0	---	---	0.0	---	0.0	---	0.0	---	---
1/2/69	36	---	4.5	---	1.3	---	0.0	0.5	0.0	---	0.0	---	0.0	---	0.5
1/9	43	---	7.0	---	---	---	4.0	17.0	---	0.2	---	0.2	---	0.0	0.3
1/16	50	---	---	9.8	14.0	---	5.5	13.3	---	0.2	0.2	---	---	---	5.0
1/23	57	0.0	---	17.5	19.8	14.8	---	36.5	0.0	0.0	---	2.0	---	---	1.0
1/31	65	---	---	3.5	17.0	10.0	6.0	0.3	0.2	1.0	---	0.0	3.0	---	2.0
2/6	71	---	---	8.3	11.5	7.0	8.0	6.8	0.0	0.0	---	0.0	2.5	---	---
2/14	79	---	---	---	---	6.0	11.0	19.0	0.0	0.0	---	0.0	4.5	---	14.5
2/20	85	---	---	---	9.5	7.5	8.0	43.5	0.0	0.0	0.0	---	3.0	---	5.8
2/27	92	---	9.0	---	7.0	7.5	8.0	29.5	0.0	0.0	---	0.0	2.0	---	1.8
3/6	99	---	---	6.0	7.5	5.5	7.5	148.0	0.0	0.0	---	0.0	3.5	---	2.5
3/13	106	---	---	25.0	28.0	28.0	32.0	8.5	0.0	0.0	---	4.0	---	---	14.0
3/20	113	0.0	---	22.0	19.0	----	38.0	11.0	0.0	0.0	---	0.0	---	5.0	14.0

		No added bacteria						
		7-1	7-2	7-3	7-4	7-5	7-6	7-7
11/19	-8	4.5	---	1.8	---	---	3.0	---
12/4	7	0.0	---	3.0	---	3.0	---	4.0
12/9	12	0.0	---	5.0	---	6.0	---	6.0
12/18	21	0.0	---	0.5	---	1.0	---	---
1/2/69	36	0.0	---	0.0	---	0.5	---	0.6
1/9	43	---	0.3	---	0.3	---	0.8	1.8
1/16	50	---	0.6	---	2.5	4.3	---	5.0
1/23	57	0.0	0.0	---	0.5	0.3	---	0.0
1/31	65	0.0	0.0	---	2.5	2.0	---	19.5
2/6	71	0.0	0.0	---	4.8	6.5	---	21.5

Table 39 - Ferrous Iron (ppm) Through Vertical Spray System (Cont.)

Date and Days After Adding Inhibitor to 2,4 & 5		Ferrobacillus sulfooxidans no inhibitor							Ferrobacillus sulfooxidans natural inhibitor						
		1-1	1-2	1-3	1-4	1-5	1-6	1-7	2-1	2-2	2-3	2-4	2-5	2-6	2-7
11/19	-8	100	---	1100	---	---	2400	---	275	---	875	---	1425	---	---
12/4	7	275	---	350	---	175	---	375	225	---	525	---	450	---	575
12/9	12	100	---	250	---	250	---	250	125	---	400	---	400	---	175
12/18	21	125	---	250	---	700	---	275	125	---	150	125	---	---	300
1/2/69	36	125	---	400	---	275	---	175	175	---	375	200	---	---	275
1/9	43	---	150	---	125	---	275	200	---	125	---	190	---	---	225
1/16	50	---	125	115	---	---	115	260	---	125	---	125	---	---	300
1/23	57	50	75	---	---	---	---	300	75	75	---	125	---	---	325
1/31	65	125	140	290	---	---	700	500	150	125	225	140	---	---	275
2/6	71	50	75	125	---	75	---	275	55	75	150	155	---	---	300
2/14	79	50	50	175	---	---	675	450	25	50	75	150	---	---	400
2/21	86	125	150	425	---	---	---	1150	---	135	150	225	---	---	550
2/27	92	125	200	775	---	---	1125	1050	150	150	150	150	---	---	450
3/6	99	50	300	625	---	---	---	1915	125	125	200	125	---	---	850
3/13	106	125	400	---	225	---	575	325	600	275	500	300	---	---	640
3/20	113	205	200	---	150	500	---	375	125	160	450	275	---	---	750

		Ferrobacillus ferrooxidans no inhibitor							Ferrobacillus ferrooxidans natural inhibitor						
		3-1	3-2	3-3	3-4	3-5	3-6	3-7	4-1	4-2	4-3	4-4	4-5	4-6	4-7
11/19	-8	---	---	850	---	1675	---	1075	100	---	1450	---	825	---	---
12/4	7	175	---	275	---	700	---	375	275	---	275	---	300	---	375
12/9	12	---	125	---	---	325	---	325	100	---	275	---	130	---	125
12/18	21	140	---	---	---	125	---	150	140	---	160	---	---	140	450
1/2/69	36	---	260	---	---	275	---	285	200	---	260	---	---	825	1425
1/9	43	---	---	---	---	150	---	350	---	135	---	650	---	400	650
1/16	50	---	450	---	---	225	---	175	175	---	225	---	---	600	700
1/23	57	---	---	---	---	250	---	300	150	75	---	75	---	---	400
1/31	65	225	290	---	---	275	---	325	75	125	---	500	---	475	500
2/6	71	---	---	---	---	200	250	300	75	290	150	275	---	---	400
2/14	79	---	---	---	---	0.1	---	28	---	---	---	14	---	---	47.5
2/20	85	---	---	---	---	425	550	900	135	---	275	---	---	525	725
2/27	92	140	525	---	200	425	---	275	125	150	---	300	---	550	400
3/6	99	---	---	---	---	150	---	400	40	125	---	275	---	300	450
3/13	106	275	---	---	---	300	---	1250	300	290	---	425	---	675	270
3/20	113	150	---	---	---	270	---	1975	125	155	---	225	---	375	300

Table 40 - Sulfates (ppm) Through Vertical Spray System

Date and Days After Adding Inhibitor to 2,4 & 5		Mixed species natural inhibitor							Mixed species no inhibitor						
		5-1	5-2	5-3	5-4	5-5	5-6	5-7	6-1	6-2	6-3	6-4	6-5	6-6	6-7
11/19	-8	---	---	1200	---	1275	---	1275	225	---	1075	---	1150	---	---
12/4	7	125	---	250	---	400	---	125	175	---	275	---	450	---	425
12/9	12	---	265	---	600	---	550	275	150	---	265	---	200	---	125
12/18	21	---	---	350	---	425	---	150	125	---	125	---	140	---	150
1/2/69	36	---	1725	---	1500	---	1225	875	110	---	100	---	260	---	150
1/9	43	---	1210	---	---	---	725	700	---	160	---	125	---	175	250
1/16	50	---	---	1300	1250	---	675	1400	---	125	125	---	---	---	250
1/23	57	---	---	---	1175	1025	---	650	75	150	---	250	300	---	250
1/31	65	---	---	400	600	425	550	270	175	175	---	175	350	---	290
2/6	71	---	---	500	550	350	350	275	150	125	---	290	350	---	---
2/14	79	---	---	---	---	6	11	19	---	---	---	---	4.5	---	14.5
2/20	85	---	---	---	1700	1000	950	725	150	220	150	---	275	---	300
2/27	92	---	750	---	1125	950	750	425	100	140	---	150	275	---	475
3/6	99	---	---	1000	1250	700	1275	1400	75	65	---	200	225	---	650
3/13	106	---	---	950	1300	1675	1550	300	125	275	---	250	---	---	300
3/20	113	225	---	400	500	---	720	260	125	150	---	125	---	500	375

		No added bacteria						
		7-1	7-2	7-3	7-4	7-5	7-6	7-7
11/19	-8	600	---	425	---	1000	---	---
12/4	7	300	---	800	---	800	---	650
12/9	12	100	---	100	---	525	---	775
12/18	21	150	---	240	---	500	---	375
1/2/69	36	125	---	250	---	525	---	400
1/9	43	---	135	---	200	---	200	350
1/16	50	---	250	---	150	275	---	300
1/23	57	225	75	---	150	325	---	275
1/31	65	225	175	---	350	425	---	475
2/6	71	115	115	---	275	400	---	425

Table 40 - Sulfates (ppm) Through Vertical Spray System (Cont.)

Date and Days After Adding Inhibitor to 2,4 & 5		Ferrobacillus sulfooxidans no inhibitor							Ferrobacillus sulfooxidans natural inhibitor							Influent H_2O
		1-1	1-2	1-3	1-4	1-5	1-6	1-7	2-1	2-2	2-3	2-4	2-5	2-6	2-7	
11/25	-2	5.9	3.3	3.0	3.1	3.4	3.8	4.0	3.2	3.1	3.3	3.5	3.8	3.8	3.7	
12/4	7	6.6	3.3	3.8	3.8	4.0	4.1	4.1	4.2	3.2	3.1	3.3	3.5	5.8	3.9	
12/12	15	7.0	3.1	4.3	4.2	3.9	3.9	5.7	6.4	3.1	4.1	4.5	---	6.0	5.5	7.1
12/20	23	6.0	3.0	3.2	3.3	3.2	3.5	3.5	6.5	3.2	3.5	3.7	---	---	---	7.2
1/2/69	36	6.3	4.7	4.0	4.4	3.6	---	4.0	6.9	3.3	3.8	4.8	---	---	3.7	7.1
1/13	47	6.5	6.3	3.5	4.2	3.2	3.4	3.6	6.8	3.8	3.9	4.6	---	---	3.5	7.1
1/20	54	5.8	5.7	3.3	---	3.1	3.2	3.2	6.8	3.7	4.2	4.1	---	---	3.4	7.0
1/23	57	---	---	---	---	---	---	2.95	---	---	---	---	---	---	2.9	
1/27	61	6.0	5.2	3.2	3.3	3.0	3.0	3.1	6.4	4.3	3.8	3.6	---	---	3.3	7.1
2/6	71	2.9	---	---	---	---	---	---	---	---	---	---	---	---	2.85	
2/13	78	5.3	3.5	3.2	---	2.8	3.2	3.1	6.1	4.3	3.6	3.6	---	---	3.3	
2/27	92	---	---	---	---	---	---	2.45	---	---	---	---	---	---	2.6	
3/6	99	---	---	---	---	---	---	2.7	---	---	---	---	---	---	2.95	
3/20	113	---	---	---	---	---	---	2.75	---	---	---	---	---	---	2.1	

		Ferrobacillus ferrooxidans no inhibitor							Ferrobacillus ferrooxidans natural inhibitor							
		3-1	3-2	3-3	3-4	3-5	3-6	3-7	4-1	4-2	4-3	4-4	4-5	4-6	4-7	
11/25	-2	---	---	3.3	3.1	3.3	5.9	3.7	4.9	3.1	3.1	3.5	3.5	3.4	3.4	
11/4	7	---	3.0	3.8	3.1	3.2	6.0	5.8	4.2	3.8	3.2	4.0	4.0	3.6	4.4	
12/12	15	6.5	4.7	---	3.5	3.9	---	5.5	3.2	3.2	3.3	6.3	4.0	4.3	5.6	7.1
12/20	23	---	3.3	---	3.5	3.9	6.6	3.6	---	4.0	3.8	---	---	3.4	3.3	7.2
1/2/69	36	---	---	---	3.5	3.7	6.6	3.6	---	4.2	4.0	---	---	3.3	3.3	7.1
1/13	47	---	---	---	3.3	3.4	4.7	3.3	6.8	4.1	3.6	5.9	---	3.2	3.1	7.1
1/20	54	---	2.9	---	3.4	3.3	4.1	3.2	6.6	---	3.6	5.9	---	3.1	3.2	7.0
1/23	57	---	---	---	---	---	---	2.75	---	---	---	---	---	---	2.6	
1/27	61	---	3.1	3.4	3.0	3.1	3.6	3.1	6.4	4.1	3.4	3.7	---	3.0	3.0	7.1
2/6	71	---	---	---	---	---	---	2.8	---	---	---	---	---	---	2.7	
2/13	78	---	---	---	3.2	3.4	3.4	3.2	4.5	3.9	3.5	3.7	3.3	3.2	3.2	
2/27	92	---	---	---	---	---	---	2.75	---	---	---	---	---	---	2.7	
3/6	99	---	---	---	---	---	---	3.0	---	---	---	---	---	---	2.95	
3/20	113	---	---	---	---	---	---	2.5	---	---	---	---	---	---	2.55	

Table 41 - pH Through Vertical Spray System

Date and Days After Adding Inhibitor to 2,4 & 5		Mixed species natural inhibitor							Mixed species no inhibitor							Influent H_2O
		5-1	5-2	5-3	5-4	5-5	5-6	5-7	6-1	6-2	6-3	6-4	6-5	6-6	6-7	
11/25	-2	---	2.5	3.0	3.0	4.5	3.4	4.5	6.7	3.0	3.5	3.4	3.5	4.8	5.0	
12/4	7	5.5	2.9	3.2	3.0	5.6	3.5	5.1	6.6	3.2	6.1	3.2	3.3	3.4	5.2	
12/12	15	---	---	4.1	3.7	3.9	4.4	---	6.6	3.5	3.9	5.2	5.1	5.8	6.0	7.1
12/20	23	---	---	3.4	3.6	3.3	4.4	3.6	7.1	4.3	6.5	5.8	3.9	6.0	6.1	7.2
1/2/69	36	---	---	3.0	3.2	3.3	3.4	3.5	7.1	5.9	7.0	6.6	3.8	4.6	4.2	7.1
1/13	47	---	---	2.9	2.9	3.1	3.1	3.3	6.6	6.8	6.7	6.8	3.6	5.5	3.8	7.1
1/20	54	---	---	2.7	2.7	3.0	3.0	3.2	6.7	6.5	6.4	5.6	3.5	4.4	3.6	7.0
1/23	57	---	---	---	---	---	---	2.85	---	---	---	---	---	---	3.0	
1/27	61	---	2.9	2.7	2.7	2.8	2.9	3.0	6.8	5.8	6.0	4.2	3.3	4.0	3.5	7.1
2/6	71	---	---	---	---	---	---	2.9	---	---	---	---	---	---	3.0	
2/13	78	---	3.0	3.1	2.9	3.1	3.1	3.2	6.6	4.5	4.3	3.9	3.4	3.6	3.4	
2/27	92	---	---	---	---	---	---	2.55	---	---	---	---	---	---	2.8	
3/6	99	---	---	---	---	---	---	2.8	---	---	---	---	---	---	3.0	
3/20	113	---	---	---	---	---	---	2.7	---	---	---	---	---	---	2.5	

		No added bacteria						
		7-1	7-2	7-3	7-4	7-5	7-6	7-7
11/25	-2	3.7	3.4	3.4	3.0	3.0	3.1	3.1
12/4	7	3.5	3.1	3.1	2.9	2.9	3.0	3.1
12/12	15	5.7	4.7	5.4	3.2	3.2	3.2	3.5
12/20	23	6.6	5.0	3.6	3.3	3.4	3.2	3.5
1/2/69	36	4.3	4.5	4.7	3.5	3.5	3.2	3.7
1/13	47	6.3	6.7	4.4	3.5	3.3	3.5	3.5
1/20	54	6.0	6.6	3.7	3.3	3.3	3.3	3.2
1/23	57	---	---	---	---	---	---	2.85
1/27	61	6.0	4.0	3.7	4.1	3.2	3.2	3.3

Table 41 - pH Through Vertical Spray System (Cont.)

TABLE 42 - TOTAL ACIDITY THROUGH VERTICAL SPRAY SYSTEM (ppm)

Date and Days After Adding Inhibitor To 2, 4 & 5		Tank No.1	Tank No.2	Tank No.3	Tank No.4	Tank No.5	Tank No.6	Tank No.7
11/19	-8	140	10	10	170	165	50	90
12/4	7	---	55	180	30	30	-2	140
12/9	12	20	20	15	-5	10	0	190
12/18	21	110	155	105	235	60	20	225
1/2	36	25	35	140	275	295	10	35
1/23	57	260	195	280	625	400	120	205
1/31	65	300	330	315	475	125	105	320
2/6	71	250	235	280	460	230	120	320
2/14	79	440	355	380	560	405	270	---
2/20	85	60	105	990	660	690	292	---
2/27	92	1475	485	255	335	230	580	---
3/6	99	2230	650	430	500	1750	510	---
3/13	106	215	485	1505	280	150	215	---
3/20	113	175	550	1075	335	145	290	---

SELECTED WATER RESOURCES ABSTRACTS
INPUT TRANSACTION FORM

1 Accession Number
W 70-05959

2 Subject Field & Group
~~[illegible]~~
05G, 05D, 05F

5 Organization
MSA Research Corporation, Evans City, Pennsylvania.

6 Title
EFFECT OF ANTIBACTERIAL AGENTS ON MINE DRAINAGES,
USE OF VIABLE ANTIBACTERIAL AGENTS TO REDUCE POLLUTION BY MINE DRAINAGES,

10 Author(s)
Shearer, Robert E. and
Everson, William A.

16 Project Designation
Grant 14010 EGJ
FWPCA

21 Note
~~Requests for report should be sent to Publications~~ Office~~, Federal Water Pollution Control Admin,~~ Wash, ~~D C 20242.~~
Available from the Clearinghouse as PB 191 215, $3 in paper copy, 65¢ in microfiche

22 Citation
Federal Water Pollution Control Administration Research Series Report DAST-21, November 1969. 138 p, 35 fig, 42 tab, 19 ref, 2 append.

23 Descriptors (Starred First)
*Acid mine water, *Water pollution treatment, *Bactericides, *Inhibitors, *Acid bacteria, Antibiotics (pesticides), Water pollution control, Mine wastes, Water quality, Water chemistry, Biocontrol.

25 Identifiers (Starred First)
Acid-bacteria inhibition, Caulobacter.

27 Abstract
The active agents in natural waters previously found to inhibit bacterial production of acid in streams were investigated in the laboratory, and inoculations of natural water previously found to be inhibitory were made, about 14,000 gallons each, to a worked-out mine in Pennsylvania. After the first inoculation, tests showed absence of inhibitory power in the water at the time it was collected, and the water used for the second test was similarly found to be only weakly inhibitory. Strains of Caulobacter were found in the natural inhibitory waters, and when adapted to acidic environments and concentrated in culture media, they induced inhibition of acid production. Other adapted strains of Caulobacters also induced inhibition. Some evidence was shown that Caulobacter inhibitors might move downstream. Four species of Streptomyces tolerated acidic conditions, and were effective against the acid-producing bacteria in test tube cultures and on solid media.
(Knapp-USGS)

Abstractor
George Knapp 3-18-70

Institution
U. S. Geological Survey, Water Resources Div.

WR:102 (REV. JULY 1969)
WRSIC

SEND TO: WATER RESOURCES SCIENTIFIC INFORMATION CENTER
U.S. DEPARTMENT OF THE INTERIOR
WASHINGTON, D. C. 20240

* GPO: 1969-359-339

RECEIVED
MAY 1 8 1970
INPUT [illegible] ON
CLEARINGHOUSE

As the Nation's principal conservation agency, the Department of the Interior has basic responsibilities for water, fish, wildlife, mineral, land, park, and recreational resources. Indian and Territorial affairs are other major concerns of America's "Department of Natural Resources."

The Department works to assure the wisest choice in managing all our resources so each will make its full contribution to a better United States—now and in the future.

MARCH 3, 1849

14010EGJ11/69